Home Office

Animals (Scientific Procedures) Act 1986

Non-technical summaries for project licences granted July - December 2021

Contents

1. Models for endocrine neoplasia 11

2. Exposure of rainbow trout to crude oil 19

3. Investigation of vitamin d metabolism in sheep 25

4. Novel approaches in bone repair 30

5. Improving treatment options for individuals with diabetes and developing intervention strategies; using beta cell replacement and regenerative therapies 37

6. Modelling cancer biology and therapy in mouse 47

7. Modelling brain tumours in the mouse 55

8. Mechanisms underpinning thrombus formation, stability and vessel repair 64

9. Developing therapeutic drugs for cancer including the establishment of tumour models 71

10. Non-invasive mucosal rodent immunisation 82

11. Normal and leukaemic blood cell development 90

12. Generation of new anti-tumour strategies for epithelial cancers 99

13. Blood clot properties in thrombosis, thromboembolism and atherosclerosis 106

14. Centrosomes and cilia in development and disease 114

15. Neuronal recording on GA Aged Animals 119

16. Infectious disease in pigs and ruminants 125

17. Regulation of gene expression in vertebrate development 136

18. Role of the inhibitory neurotransmitter naag and its transporter in pathologies involving glutamatergic dysfunction 142

19. Cellular electrophysiology of cardiac arrhythmias 151

20. Development of combined treatments for cancers 158

21. Control of blood flow and cell function in the brain and other organs, in health and disease 168

22. Assessment of biological response to novel ocular drug delivery formulations and devices 176

23. Provision of services to investigate novel health technologies 185

24. Induction of anti-tumour immunity 197

25. Immune regulation at mucosal and barrier surfaces 205

26. Neuron-glia biology in neurodegenerative disorders 214

27. Immune-based treatments for cancer 221

28. Cell fate determination and morphogenesis during early mammalian development 229

29. Synapses in health and disease 240

30. The nervous system influences on pancreas development and physiology 254

31. The pathogenesis of a neglected tropical skin disease 261

32. Understanding and treating preterm birth related pregnancy disorders 270

33. Gamma delta t cells and body surface immunity 280

34. Developing anti-malarial transmission blocking interventions 292

35. The chicken chorioallantoic membrane (cam) assay for biomaterial testing in tissue engineering 301

36. Development of novel therapy strategies for skin diseases 312

37. The role of transposable elements in cichlid fish development and evolution 321

38. Understanding the mechanisms and mediators involved in adverse pregnancy outcomes 330

39. Understanding trait variation in migratory birds 339

40. Developing flare-responsive intra-articular steroid injection 345

41. Investigating mechanisms and novel therapeutics for critical limb ischaemia 355

42. Visual processing in frogs 365

43. Production of normal blood 371

44. Breeding and maintenance of genetically altered and germ free mice 378

45. Molecular imaging and radionuclide therapy research for cancer 387

46. Minimising tissue damage in electrosurgery 395

47. Role for xxx in gene regulation 401

48. Brain development and neuronal network maturation 408

49. Airway fibrosis 415

50. Hypoxia, carbon dioxide and acid sensing in respiratory chemoreceptors. 422

51. Assessment of bioaccumulation in fish 428

52. Translation of the immunological synapse 435

53. Control of worm infection by the immune response and drugs 443

54. Defining immune mechanisms that promote inflammatory arthritis and associated co-morbidities to identify new therapeutic targets. 451

55. Development of vector control strategies and intervention methods concerning the spread of malaria 460

56. Novel drugs for intractable cancers 469

57. Establishing the pathophysiological impact of cigarette smoke extract in mice as a model for chronic obstructive pulmonary disease (copd) 478

58. Improving our understanding of the bovine immune system 491

59. Defining osteoarthritis mechanisms 502

60. Understanding genetic and diet effects on the productivity and environmental footprint of dairy cattle. 510

61. Investigation of drug resistance mechanisms in haematological malignancies 517

62. Imaging and treating tumours 525

63. Novel nanomaterials as delivery vehicles for drug and cellular therapies 533

64. Technical development of refined phenotyping assays. 544

65. Antibodies for research and clinical purposes 556

66. Study and manipulation of the tumour immune microenvironment for cancer therapeutics 564

67. Investigating tick-borne bunyavirus interactions with ticks and how these interactions affect transmission and pathogenesis of viruses in a small animal model 573

68. Neural mechanisms of pain control 582

69. Immunotherapy of amyloidosis 590

70. Strategies to improve donor organ function in a porcine model 601

71. Investigating immune responses in mice 606

72. Generating new models of human cancer 613

73. Signalling in normal and abnormal blood cell function 620

74. Targeted tumour therapy 628

75. Understanding how climate change affects mammalian immunity and control of infection 641

76. Early safety assessment, investigatory and efficacy studies 649

77. Cellular and molecular mechanisms of the mouse embryo development 664

78. Tumour suppression and therapy 676

79. The effects of aging and prior injury on tissue regeneration and fibrosis 686

80. Zebrafish models to identify determinants of outcome in immune-mediated disease 696

81. Antibody production for clinical and research applications 702

82. Immune signalling in infection and cancer 710

83. Cancer and the microenvironment 721

84. Vascular regulation of fibrotic disease 728

85. Environmental adaptation in fish 737

86. Dna double-strand break repair, immunity & cancer 744

87. Mechanism of action of vaccines and adjuvants 751

88. Impact of drugs and environment on the brain through the lifespan 757

89. Role of protein degradation in health and disease 764

90. The integrated stress response in respiratory disease 771

91. Phenotypic plasticity and adaptation of cichlid fish in response to novel environments 782

92. The impacts of marine engineering on wild fish 790

93. Development of live bacterial therapeutics for the treatment of cancer 798

94. The role of nitrite in coronary artery disease and its associated risk factors 806

95. Modulating the immune response in cancer 816

96. Trans-generational effects of age in birds 825

97. Investigation into the inflammatory mechanisms associated with inflammatory bowel disease and intestinal cancer 835

98. Cell surface receptor functions in fertility 843

99. Cell biological pathways in neurodegeneration 849

100. Exploring the role of the cell surface molecule podoplanin in fibroblast-macrophage cross talk in arthritis 858

101. Genetic analysis of metastatic cancer 869

102. Host-pathogen interactions and vaccines 876

103. Investigating the mechanisms driving cardiac fibrosis and its effects on cardiac function 883

104. Treatment of chronic inflammatory disease 891

105. Determination of cns efficacy and safety pharmacology 897

106. Therapeutic potential of tregs 907

107. The ecophysiology of fish 915

108. Immune mechanistic models of autoimmune disease 921

109. Mechanisms of cardiometabolic disease 927

110. Developing and studying novel mouse models of glioma development and progression 943

111. Creation and repair of ano-rectal fistulae in a porcine model 951

112. Tissue function and metabolism in heart disease 958

113. Preclinical evaluation of cancer therapeutics 966

114. New therapeutic strategies to treat chronic pain 976

115. Pathways of filopodial formation 989

116. Standards in virology 996

117. Ecology and epidemiology of infectious disease in wildlife 1010

118. Mechanisms of metabolic regulation in health and disease 1017

119. Neural circuits in health and disease 1028

120. Tau pathology in alzheimer's disease 1035

121. Supply of materials to develop and maintain tests for transmissible spongiform encephalopathies 1047

122. Immunotherapy and vaccine development for cancer and sars-cov-2 1054

123. Studying host interactions in tumour and metastasis 1061

124. Developing treatments for huntington's disease 1069

125. Mouse models of chronic inflammatory clinical conditions. Word document missing 1076

126. Colorectal cancer initiation and progression 1088

127. Tuberculosis immunity and vaccine development 1095

128. Impact of hormones and inflammation on reproductive health and disease 1103

129. Prevention and treatment of sepsis 1112

130. Synaptic and circuit mechanisms for neural computation 1119

131. Improving prognosis from vascular dementia 1125

132. Evaluation of cancer therapies in orthotopic cancer models 1133

133. Production of antibodies, antisera and blood products iii 1140

134. Investigating the immunology of cancer and the development of cancer therapeutics 1149

135. Isolation and propagation of virus in eggs 1156

136. Developing non-viral gene therapies 1164

137. Molecular mechanisms of t cell mediated immune responses 1173

138. Understanding immune cell development and its functional consequences 1183

139. Mechanisms of cardiac arrhythmogenesis – insights from murine model 1190

140. Modelling gene therapy for congenital bladder dysfunction 1201

141. Metabolism as a novel target for cancer therapy 1208

142. The role of morphological and genetic abnormalities in tumour progression 1217

143. The use of immunological tools for monitoring farm cattle health and prevalence of bovine tuberculosis (btb) 1228

144. Production, breeding & cryopreservation of genetically altered mice 1236

145. Discovery of cancer genes and assessment of their therapeutic potential 1241

146. Molecular basis of neurodevelopmental disorders 1251

147. Acute and chronic kidney disease: translational studies using the pig 1257

148. Reproductive toxicology 1268

149. The reciprocal interaction of inflammation and cancer 1277

150. Nucleic acid sensing and interferon-regulated genes 1284

151. Breeding, production, archiving and the application of assisted reproductive techniques of genetically altered mice 1292

152. Mechanisms of neural circuit development in health and disease conditions 1299

153. Development of an implantable device for the treatment of brain tumours 1306

154. Understanding epithelial tumour initiation 1316

155. Imaging neurons to understand memory formation and loss 1326

156. The biology of body weight and body composition 1334

157. Repair and protection of the retina and optic nerve 1343

158. Analysis of skin carcinogenesis in transgenic mice 1354

159. Mechanisms regulating neural stem cell activity and neuronal production 1362

160. Information processing in neural circuits 1369

161. The regulation of platelet function as a central mechanism for the control of cardiovascular function and development 1376

162. Molecular determinants of brain development 1390

163. Neuronal and glial function and dysfunction across the life-course 1399

164. Understanding the mechanisms of the carotid body hyperactivity caused by chronic hypoxia 1406

165. Regulation of immune responses in cancer 1418

166. In vivo pharmacokinetic and pharmacodynamic properties of potential new treatments for cns and other disorders 1427

167. Brain networks in epilepsy and neurodevelopmental disorders 1435

168. Towards mechanistic understanding and improved treatment of nervous disorders 1441

169. Lymphocyte development and ageing 1452

170. Gene and environment influences contributing to psychiatric disorders 1460

171. Studies on the neurobiology of sensation 1469

172. Production of genetically altered animals and provision of tissues (service provision) 1484

173. Preclinical validation of intraperitoneal delivery of chemotherapy for treatment of ovarian cancer 1492

174. Understanding the neuronal network involved in the regulation of glucose metabolism, feeding behaviour and energy expenditure 1500

175. Target investigation and characterization of potential medicines 1510

176. In vivo assessment of novel injectable hydrogel biomaterials for spinal disc repair 1519

177. Vascular calcification in kidney dysfunction 1527

178. Environmental stresses in forages affecting ruminant digestive physiology 1536

179. Models for experimental therapeutics of childhood cancers 1542

180. Understanding and developing new treatments for neurological diseases 1549

181. Alternative pre-mrna splicing control and its contribution to tissue injury and pain 1559

182. The physiological roles of clathrin diversity 1569

183. Breeding and maintenance of genetically altered mice 1578

184. Glymphatic system in the ageing brain 1586

185. Immune responses in tumour development 1593

186. Central nervous system control of physiological functions 1600

187. Neuronal circuits regulating metabolic and cardiovascular health 1607

188. Analysis and therapy of cerebellar disease 1616

189. Vascular protective genes in angiogenesis 1624

190. Role of natriuretic peptides in cardiovascular homeostasis and disease 1636

191. Vaccine candidates for group a streptococcus infections 1644

192. Novel mechanisms of co2 signalling in the physiological systems 1649

193. Nutrition of growing and mature ruminant livestock 1657

194. Investigation into the mechanisms causing acrodysostosis type 2 and related therapies 1665

195. Neural mechanisms of visual cortical plasticity 1672

196. Immune and inflammatory mechanisms of cardiovascular disease 1679

197. The aberystwyth liver fluke sample-bank for the evaluation of diagnostic tests 1688

198. Analysis of thymus development and function 1697

199. Neuronal mechanisms of appetitive learning 1704

1. Models for endocrine neoplasia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to increase our understanding of the biology of endocrine tumours, and to develop novel treatment strategies for them. This is because current treatments are largely ineffective, which is partly due to a lack of knowledge regarding the molecular mechanisms driving the development of these tumours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Current treatments for endocrine tumours, particularly those of the pancreatic islets and pituitary are limited, meaning there is a clinically unmet need for the identification of novel drugs, or therapeutic approaches. Therefore, this project is important for increasing our understanding of the molecular biology of endocrine tumours, which will identify novel pathways, and or genetic targets in these tumours, which may ultimately be targeted by novel or existing drugs. In addition, the assessment of the efficacy of any candidate novel therapeutic is important to provide evidence of its potential use in the clinical setting.

What outputs do you think you will see at the end of this project?

Overall, with this project we aim to increase our knowledge of the biology of endocrine tumours. This includes increased understanding of the genetic, and epigenetic pathways involved in the development of pituitary and pancreatic neuroendocrine tumours.

Within this study we also aim to develop and characterise new mouse models based on human genetic studies.

In addition, we aim to explore the use of epigenetic targeting drugs, and other molecules for treatment of these tumours. This will include the development of novel agents, as well as repurposing agents that are already used in the treatment of other cancers, or diseases.

Therefore, our outputs may include:

publications in good quality peer-reviewed journals, as well as abstracts at national and international meetings new mouse models of endocrine tumours novel anti-tumour agents

Who or what will benefit from these outputs, and how?

Our studies will help to understand genetic causes, cellular pathways and the interactions between different organs, in diseases underlying endocrine tumours. In addition, our phenotyping studies may uncover clinical symptoms that have not yet been investigated in patients. In the short term this information may therefore be used to direct future endocrine tumour research, as well as identify new clinical investigations that should be undertaken in endocrine tumour patients.

Our studies will also help to identify novel drugs, or other agents that could be used for the treatment of endocrine tumours in patients. In the long term our pre-clinical studies may therefore provide evidence for future clinical trials testing drugs that are novel, pre-existing or repurposed

Beneficiaries of our project may therefore include: scientists and other researchers clinicians patients

How will you look to maximise the outputs of this work?

To maximise the output of our work, both positive and negative results will be published in peer- reviewed open-access journals. In addition, work will be presented at national and international conferences. Details of unsuccessful approaches will also be disseminated through public databases

e.g. publications submitted to pre-print servers including bioRxiv.org.

Collaborations will be established with international groups, and novel mouse lines will also be made available through archives, for example European Mouse Mutant Archive EMMA.

Species and numbers of animals expected to be used

- Mice: 9500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our studies are utilising predominately adult mice. This is because we are studying the biology, and potential treatments for endocrine tumours, which most commonly develop during adolescence and adulthood in patients. These tumours secrete hormones, for example insulin and prolactin, and therefore can effect metabolic pathways other than those directly occurring at the tumour site. In addition, our research focuses on genetic disorders, for example Multiple Endocrine Neoplasia Type 1, in which patients develop tumours simultaneously in different organs, for example the pancreas and pituitary. It is therefore important to look at whole organism effects, for which mice represent an model that closely recapitulates the human disease.

Typically, what will be done to an animal used in your project?

A typical experience for a mouse on this project would be: aged to 7 months, provide a blood sample from a superficial vein receive twice weekly intraperitoneal injection of an anti-cancer drug for 4 weeks daily administration of BrdU in drinking water for 4 weeks fasting for 4h provide a blood sample from a superficial vein killed by a schedule 1 method

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects due to endocrine tumour development may include, but are not limited to: Alterations in balance and/or walking (<0.5%)

Reduced mobility and/or feeding due to tumour effects (<2%)

Increased urination or drinking due to disturbances in glucose metabolism (~10%)

Weight loss and loss of condition due to metabolic disturbances, e.g. due to insulin secretion from pancreatic neuroendocrine tumours (<5%)

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity of this licence is expected to be moderate. The majority of mice bred under this licence will be tumour bearing (>60%), and therefore have the potential to experience adverse effects. These adverse effects will be monitored closely to ensure they are not prolonged, or severe, and mice will be culled as soon as the detailed humane endpoints are met.

Control mice that do not develop tumours will also be bred (<30%), however they may be used in phenotyping and drug studies, within which they may also meet the moderate severity due to procedures including repeat imaging (<5%) and administration of novel anti-cancer agents (<5%).

It is expected that (~20%) of mice will be controls that will only be used as breeders and therefore will be sub-threshold.

In summary, the expected severities of the mice wil be: moderate (60%); mild (20%); subthreashold (20%).

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are studying the biology, and identifying novel treatments for endocrine tumours. These tumours secrete hormones, for example insulin or prolactin, and therefore can effect metabolic pathways other than those directly occurring at the tumour site. In addition, our research focuses on genetic disorders, for example Multiple Endocrine Neoplasia Type 1, in which patients develop tumours simultaneously in different organs, including the pancreas and pituitary. It is therefore important to look at whole organism effects, for which mice represent a model that closely recapitulates the human disease.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of cell culture models, and are striving to develop primary cell models that may be able to replace some mice in some preliminary studies. For example, we are trying to immortalise and grow cells taken from mouse organs in the laboratory, and these could be used to test whether new drugs might be effective, before they are tested in live mice. In addition, we will assess potential therapeutic agents in already established cell lines before undertaking mouse studies.

Why were they not suitable?

We have been successful in establishing primary parathyroid cultures, and pancreatic islet cultures in preliminary studies, however these cells are not viable for long periods in culture and do not proliferate, as they do in vivo. Therefore, the assessment of novel therapeutic agents is limited as one of the measures of efficacy is reduction in proliferation. In addition, it is not possible to assess the effects of treatment, and hormone secretion on non-tumour cells and organs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers are based on estimates from previous years performing similar research studies to address similar questions and numbers of test agents.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animals are only bred as required to supply animals for experimental requirements. The number of animals required for the experimental studies is determined by power calculations to achieve an effect size. The group is in contact with our establishment's statistics department who offer advice on statistical requirements. We may also use online tools e.g. experimental design assist (https://eda.nc3rs.org.uk), and regularly check the nc3rs website for alternative approaches.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In all our mouse work, we use statistical analysis to ensure that the minimum number of mice are bred for the study, and that we use only the number of mice that are required to produce meaningful and useful results in order to answer the experimental questions. We are able to study the effects of drugs in multiple organs within an individual animal, for example in mice which develop tumours in more than one organ. Similarly, we can image the same mouse several times to study the development of organs or tumours, rather than using several mice once. In addition, we will use tools for colony management (e.g. MCMS) to ensure we do not overbreed and waste animals. We are also trying to establish cells from mice that will grow long-term in the laboratory. These could be used to replace some mice for the early stages of testing of new drugs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have previously established and characterised mouse models for use in this study and further models of endocrine tumours, based on human diseases will be established using our prior experience with techniques and facilities that we have readily available. Our conditional Men1 models are particularly useful for assessing the effects of drugs since unlike the conventional Men1 knockout models, all mice develop pancreatic tumours, and all tumours develop at the same age, allowing for more accurate comparisons between treatment groups. Thus, the age of tumour development is more defined and the organs in which they will develop; thereby making it easier to predict when the mice may begin to have symptoms that could cause distress. Furthermore tumours can develop as early as 5 months, therefore reducing the length of the protocol.

Why can’t you use animals that are less sentient?

Mice will be used in our studies and were chosen as they represent the lowest mammal, displaying a sufficiently similar endocrine system, and genetic similarity to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our team has developed expertise and experience in mouse welfare, and have refined our tests to ensure that the highest quality data is generated for the least welfare cost. We are also keen to minimise severity and increase the welfare of these animals. To ensure this, we will use non-invasive tests that only cause temporary discomfort where possible. For

administration of drugs, a small pilot study will be undertaken for new drugs, with increased cage observations and welfare checks to ensure that the drug is safe. We also aim to use long acting drugs where possible to reduce the frequency of dosing. During every test, mice are closely observed and anaesthetics or analgesics used when appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will conform to the ARRIVE guidelines on animal studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We attend termly gold standard animal welfare meetings at which a University representative of the 3Rs gives regular updates. We also consult the 3Rs and LASA websites for techniques and alternative models. In addition, we will have regular discussions with the animal technicians, and NACWO, as well as other researchers, to share experience on new, more refined techniques.

2. Exposure of rainbow trout to crude oil

Project duration

5 years 0 months

Project purpose

- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Fish, Crude oil, sensory, taint

Animal types	Life stages
Rainbow trout, oncorhynchus mykiss	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To maintain an expert panel, trained in sensory assessment, to detect hydrocarbon taint in fish and shellfish in the event of a major oil spill. The project provides a set of reference materials – rainbow trout exposed to oil over a set period of time varying in PAH (polycyclic aromatic hydrocarbon) concentrations – required to monitor panel performance and train new assessors.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Contaminants can enter the natural environment through anthropogenic (human) activities and accumulate in the tissues of fish and shellfish and in severe cases, these contaminants can present a risk to human health. Some contaminants such as polycyclic aromatic hydrocarbons (PAH's) can be detected as a taint in the foodstuffs. The project results in the production of a reference material for training and performance monitoring of an expert sensory taste panel trained in the detection of hydrocarbon taint in fish and shellfish. The sensory panel provides a rapid and sensitive method for detecting taint

following pollution incidents allowing the Government to take action, closing Fisheries where necessary, preventing potentially contaminated food reaching the commercial market. Thus protecting the general public from potentially contaminated fish and shellfish.

What outputs do you think you will see at the end of this project?

This project aims to ensure that a panel of expert sensory assessors retain their expertise and perform taint assessments which produce reliable and robust data. Recruitment and training of new assessors is an essential part of the project to maintain a pool of trained assessors.

Who or what will benefit from these outputs, and how?

Taint assessment provides a rapid and sensitive method for detecting taint following pollution incidents. This may be considered as a screening method prior to chemical analysis which would be used to confirm the presence of contamination. This allows swift action to be taken, closing Fisheries where necessary, preventing potentially contaminated food reaching the commercial market. Human health is protected and the reputation of the seafood and aquaculture industries safeguarded reducing the potential to damage the economy following a pollution incident.

How will you look to maximise the outputs of this work?

This project aims to ensure that a panel of expert sensory assessors retain their expertise and perform taint assessments which produce reliable and robust data. Recruitment and training of new assessors is an essential part of the project to maintain a pool of trained assessors.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rainbow trout (Oncorhynchus mykiss) are used they are a farmed species available in the size and numbers available for the project. Farmed trout offer a consistency in raw material over time as they can be obtained from the same farm where conditions are controlled with respect to diet, water quality, disease and size. Rainbow trout can tolerate a high stocking density and show no signs of distress when moved to a smaller tank.

Typically, what will be done to an animal used in your project?

The project exposes live rainbow trout to crude oil (2.5ml per 100 litre tank) over a period of exposure times. A maximum of 7 fish per tank, equivalent to a stocking density of 28 kg/m3, are exposed for up to 4 hours at a temperature of 15 °C (± 1 °C).

What are the expected impacts and/or adverse effects for the animals during your project?
Rainbow trout can tolerate a high stocking density and show no signs of distress when moved to a smaller tank. No adverse effects recorded in previous studies, experience to date suggests that the fish tolerate the oil exposure without any apparent clinical signs

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity category Mild for all animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Taint which appears in the fish muscle, is a result of the oil entering the fish through the gills and skin from the water. The intensity of taint resulting from fish exposed to crude oil will vary according to the length of time the fish is exposed to the oil. Spiking a sample (fish fillet) with crude oil will not give an accurate representation of taint which would naturally accumulate in a live animal exposed to the same oil as the whole oil would be present in the sample.

Which non-animal alternatives did you consider for use in this project?

Chemical analysis by GC-MS (gas chromatography mass spectrometry) Protein assay to look at the biological effects of exposure to PAH contamination.

Why were they not suitable?

Chemical analysis cannot replace the use of live animals for taint analysis as it is a sensory procedure (determined by smelling and tasting). Chemical analysis is a lengthy analytical procedure, typically a maximum of 10 samples can be analysed with results available after 3 days. In the event of an oil spill an expert sensory panel can rapidly assess many samples (40-50 per day), giving a rapid response to the potential contamination of a fishery. Swift action can be taken by authorities to close the fishery and prevent contaminated fish being taken and reaching the food chain, thus protecting human health.

Protein assay requires tissues to be sampled from live fish. In the event of an oil spill sampling would have to be conducted by highly skilled/trained personnel. Specialist equipment and storage facilities would also be required (tissue samples to be kept at -80

degrees C prior to analysis). It would not be practical or possible to adhere to scientific procedures following an oil spill.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of fish used is based on providing sufficient material (fish muscle) required to run a taint panel every 2 months. A panel will typically consist of between 7-12 assessors, each assessor is required to taste each sample. Within each training session a range of samples are presented ranging from absence of taint to extremely high taint. This is achieved by exposing live fish to crude oil over a period of time. A suitable range of intensities can be achieved by exposing fish for periods of time extending from 20 minutes to 4 hours. Each year the project requires 72 fish, of these 56 are exposed to crude oil and the remaining 16 unexposed and used as controls.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The concentration of PAH in the fish flesh is required to reach the detection and recognition threshold of the stimulus (oil taint) for 50 % of the assessors to ensure taint is detected.

Previous experimental trials have shown that the relationship between taint score and PAH (polycyclic aromatic hydrocarbons) concentration is optimised at the exposure intervals used for the project, thus ensuring the desired results can be achieved using fewer exposure times leading to fewer animals being required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Previous experimental trials have shown that taint intensity reaches a plateau after 8 hours. The minimum number of exposure periods has been selected to provide tainted material over a range of intensities (absent to extremely strong).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rainbow trout (Oncorhynchus mykiss) are used as they are a farmed species available in the size and
numbers available for the project. Farmed trout offer a consistency in raw material over time as they can be obtained from the same farm where conditions are controlled with respect to diet, water quality, disease and size. Rainbow trout can tolerate a high stocking density and show no signs of distress when moved to a smaller tank. No visible signs of stress have been recorded during the exposure period.

Why can't you use animals that are less sentient?

Animals are required to be of a size large enough to provide sufficient tainted material for assessment by a panel of expert assessors. Rainbow trout are shown to tolerate conditions such as high stocking density.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedure has a severity rating of mild and all animals are euthanized at the end of the trial. Animals are observed at regular intervals throughout the 4 hour procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Refer to the current Government legislation – ASPA

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Refer to the current Government legislation - ASPA

3. Investigation of vitamin d metabolism in sheep

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants
    - Improvement of the welfare of animals or of the production conditions for animalsreared for agricultural purposes

Key words

Sheep, Vitamin D, Rickets, Supplementation, Trial

Animal types	Life stages
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to define the best way to prevent vitamin D deficiency in sheep.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We have recently discovered that many sheep develop severe vitamin D deficiency during the winter in Scotland. In some cases this can lead to painful and debilitating skeletal complications including rickets. We propose to undertake an objective study to define the best way to prevent the development of hypovitaminosis D in extensively farmed sheep during the winter.

What outputs do you think you will see at the end of this project?

We expect this programme of work to allow us to define a strategy to prevent hypovitaminosis D in sheep during the winter period where there is an absence of UVB light in temperate regions. We will disseminate our findings in a peer reviewed manuscript. We will also share our results via Scottish farming networks through regional talks and seminars.

Who or what will benefit from these outputs, and how?

The principal beneficiary of this work will be sheep living in temperate regions since we anticipate that our work will define how they can be protected from the development of low vitamin D status and its associated metabolic complications. Through the improved welfare and health of the sheep in temperate regions over the winter period, this project will also boost the wider farming sector through improved productivity and profitability of their farming activities.

How will you look to maximise the outputs of this work?

We will disseminate this work widely through a scientific publication, talks and outreach activities as exemplified in our previous work on vitamin D metabolism in other species.

Species and numbers of animals expected to be used

- Sheep: 180

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying sheep because they are the animals which develop low vitamin D status over winter and it is sheep whose health and welfare we are seeking to improve.

Typically, what will be done to an animal used in your project?

The sheep will be given either an injection or oral bolus of vitamin D and will have their vitamin D status assessed before and after the intervention through the measurement of vitamin D metabolites in serum samples harvested at regular timepoints post vitamin D administration.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not envisage any serious side effects during this project. The only potential complication is hypervitaminosis D and associated hypercalcaemia but at the doses of vitamin D used, we do not expect this to happen. If a sheep in a control group develops any clinical evidence of metabolic bone disease, it will be immediately withdrawn from the

study and administered vitamin D. If an adverse event occurs we expect all to be mild in nature.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect to have any side effects beyond standard minor complications of blood sampling such as localised bruising at the venepuncture site. If these occur all will be mild. Although technically possible, we do not expect hypervitaminosis D and associated hypercalcaemia to occur. If this does then the complication is likely to be transient and mild. Again, although technically possible, we do not expect any of the control, untreated sheep to develop rickets during the lifetime of the experiment. If this complication occurs it is likely to be mild since it will be detected early due to close monitoring of sheep and we envisage that our early results will define how best to boost vitamin D status in sheep. We plan to begin this work in the autumn and measure 25(OH)D ASAP after sample collection. We then hope to be able to treat any remaining sheep with the most appropriate vitamin D supplementation approach ahead of the timepoint when clinical signs of rickets emerge. Therefore, we expect that the risk of the untreated sheep getting clinical skeletal disease is very low in our experimental approach and even if this does occur, it will be detected early once clinical signs are mild.

What will happen to animals at the end of this project?

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are seeking to establish how best to prevent hypovitaminosis D in sheep and there is no meaningful way this can be explored other than by studying vitamin D metabolism in sheep.

Which non-animal alternatives did you consider for use in this project?

There are no meaningful non animal alternatives available to us which could allow us to address the research aims of this project.

Why were they not suitable?

There are no meaningful non animal alternatives available to us which could allow us to address the research aims of this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to undertake a single experiment at two different sites. In each experiment we plan to have 30 control, non supplemented sheep, 30 sheep administered with oral vitamin D and 30 sheep administered with injectable vitamin D. We have allocated 30 sheep to each group based on a power calculation which utilises data from our previous studies. Based on a mean 25(OH)D concentration of 7.9nmol/l at the start of the study and a standard deviation of 4.3nmol/l and with 90% power and at 5% significance, we require study groups to each contain 30 sheep to be able to detect an increase to 11.6 nmol/l. This experiment will be undertaken on two Scottish flocks. Consequently, the total number of animals which will be used is 180 (90 in each experiment).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We utilised historical data from near identical populations of animals to guide a power calculation to define the minimum number of animals needed for each study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have used a power calculation using objective, high quality and relevant data.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will study sheep and will simply be administered vitamin D in a manner that is routinely done as part of standard management practice, namely through injection and oral drench. The only variation from standard veterinary practice is that we will assess vitamin D status objectively through the measurement of serum 25 hydroxyvitamin D (25(OH)D) concentrations before and at several time points post vitamin D administration.

Why can't you use animals that are less sentient?

We can only use sheep since these are the animals in which we are keen to prevent hypovitaminosis D in during winter periods in temperate climates.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will only be administering injections or boluses containing vitamin D and taking blood samples. These are all minimally invasive techniques that are standard management practices. The blood samples will be collected by experienced colleagues who are experienced in venepuncture techniques.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be following standard, well established techniques for collecting blood samples in sheep as well as the administration of injections and oral drenches.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I regularly receive information about the 3Rs from named veterinary surgeons and the wider veterinary and research community.

4. Novel approaches in bone repair

Project duration

5 years 0 months

Project purpose

- Basic research
  - Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Bone, Healing, Materials, Anabolic agents

Animal types	Life stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Surgical approaches to bone healing have remained largely unchanged for decades. This project explores how bone healing can be improved and accelerated by testing new agents/approaches to stimulate bone regeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Bone healing is a lengthy process resulting in reduced mobility which directly impacts everyday life at work and home. This work aims to provide new information toward helping individuals recover from a bone injury quicker, and better, to allow them to regain independence and mobility as soon as possible.

What outputs do you think you will see at the end of this project?

This project will provide new information on how the bone regeneration healing process can be accelerated to improve repair and reduce bone healing times. Through the study of our new drug, we hope to show how treatment with this agent stimulates bone healing.

Who or what will benefit from these outputs, and how?

In the short term, the research community will benefit from the validation of a new and rapid approach to testing agents which might stimulate bone repair, and which we will disseminate via research publications, conference proceedings and through institutional channels supporting 3Rs. Furthermore, those suffering from bone fracture or the need to combat accelerated bone loss will also benefit if improved understanding of bone repair is achieved, and the further development of our new bone targeting agent, to treat this problem.

How will you look to maximise the outputs of this work?

The findings from this work will be maximised through the dissemination of data describing this new model system and bone-targeting agent. This will be achieved through typical channels including publications and conference proceedings (with a focus on bone e.g. Bone Research Society, European Calcified Tissue Society). This will be conducted by both collaborating institutions on this project.

Species and numbers of animals expected to be used

- Mice: 1800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice represent the lowest mammalian option for us to study bone regeneration, and which have a mature skeleton resembling that of humans.

Typically, what will be done to an animal used in your project?

Normal animals or animals with a genetic modification related to bone and joint biology will be used. Genetically modified animals may have moderate skeletal defects (e.g. high/low bone mass, longer/shorter bone length). Animals will have a small hole (osteotomy) created in their bones, which represents a small fracture. Test agents which might accelerate the rate at which this bone defect/fracture heals will be given, and the bones monitored during the healing period.

Specifically, experimental animals not used for breeding purposes animals (approx 50%) will undergo general anaesthesia (up to 30 mins), bone defect/osteotomy surgery (in the tail vertebrae or long bone (hindlimb)), administration of agent (typical x28 injections), weekly imaging, dynamic weight bearing analysis (optional for long bone osteotomy groups only), injections of fluorescent agents to visualize bone formation rates (x2 injections), and will be killed up to week 8 via approved methods (including withdrawal of fluids e.g. blood).

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that most animals undergoing surgery will experience a low level of pain shortly after the procedure which will be managed with aseptic technique using sterilised instruments and materials to control for infection, monitoring and pain relief (analgesics).

A minority of animals may show complications in the short term. This might include: wound breakdown - bleeding immediately following surgery will be treated with immediate compression and repair of the wound with surgical glue applied externally. Further breakdown of the wound will be cleaned and re-closed once.

lameness - animals will receive appropriate pain relief prior to surgery and examined at 6-8 hours to ensure that they are pain-free. If animals continue to exhibit lameness or signs of pain, a repeat dose of analgesia will be administered and moist food will be placed on floor of cage. Animals will be inspected, examined and weighed daily until 4-7 days post op, with ongoing inspections and examinations at least twice per week.

The above impacts/adverse effects will be addressed as described for up to 24 hrs before humane end points (below) are actioned.

Breathing problems may occur following inhalation anaesthesia - listlessness or laboured respiration in animals following anaesthesia will be closely monitored during recovery upon a heated stage to maintain body temperature. Animals are typically expected to recover from anaesthesia within 10 mins. If animals are not recovered by 30 minutes humane end points will be actioned

Humane endpoints for this step:

Weight loss of more than 15%

Piloerection, hunched posture

Infection (as inflammation, swelling, redness) or successive breakdown at wound site or tail necrosis

Lameness and listlessness for more than 24hours

Tail Necrosis

Laboured respiration particularly if accompanied by nasal discharge/cyanosis

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity is expected in animals undergoing osteotomy surgery (50%). Mild or sub-threshold severity for control mice and those used for breeding.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Modelling the complex inorganic and organic components of the bone environment, including the cellular and matrix protein aspects, is extremely challenging. Furthermore, it is impossible to mimic the response of this environment to trauma and accurately model the impact of different cell types and matrix proteins. This proposal has used non-animal models to prove that a new agent aimed at improving bone healing is able to stimulate relevant cells in laboratory tests. This evidence supports the use of this agent as a potential therapy to improve bone regeneration and healing. However, it is impossible to determine this without the use of animal models.

Which non-animal alternatives did you consider for use in this project?

We have previously used in vitro cell culture to assess the effect of our new agent, and where a strong stimulation of bone formation and reduced numbers of bone destroying cells were seen. We have also considered the use of ex vivo model systems to address this question, but which are only capable of modelling limited elements of the bone repair process. These include the use of multi-cell cultures and human bone samples.

Why were they not suitable?

Multi-cell cultures such as 3D matrigel cultures or the use of transwell inserts in 2D, do not account for matrix contributions, and human bone samples do not culture well and where the impact of trauma cannot be easily accounted for.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used specific mathematical calculations based upon previous studies and the likelihood of our agent producing positive results, to estimate the number of animals we will use in our study. For all experimentation, the lowest possible number of animals will be tested whilst ensuring that the experimental result is robust.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used statistical analysis to calculate the minimum number of animals necessary for this project. In addition, longitudinal weekly imaging of the same animal over the healing phases will be used to reduce the need for multiple animals at different time points.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have used statistical analysis to calculate the minimum number of animals necessary for each experiment within this project. Furthermore, pilot studies will be used to further determine bone healing rates and which will inform further calculations of necessary animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

- Which animal models and methods will you use during this project?
- Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in a i) long bone and ii) tail vertebrae osteotomy model. This species and procedures have been chosen as they represent the lowest animal model possible to study bone regeneration, and where a modified version of bone fracture will be tested. This offers the opportunity to significantly reduce suffering and harm to the animal induced by unnecessary and complex surgical procedures.

The exploration of a tail vertebrae osteotomy model to clinically measure bone regeneration is anticipated to cause less distress to animals as the proposed defect is considerably smaller than those used in full fracture models, the necessary surgery is considerably less as is the need for external fixators to support the bisected bone. Furthermore, the overall systemic impact of the defect upon the whole body is less due to the remote site of injury and significantly reduced necessity of tail vertebrae compared to long bones (e.g. femur). Although a small degree of pain and discomfort associated with this procedure is anticipated, it is expected to be a considerable reduction versus that of a long bone fracture, and where similar scientific information might be generated.

In addition, animal handling will be kept to a minimum and where the scoop and lift technique will be used, particularly with regard to protecting the tail following tail osteotomy surgery.

Why can’t you use animals that are less sentient?

Less sentient animals do not possess the same sort of skeletal structure. Small rodents are the lowest mammals which can be used to recapitulate human bone healing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be monitored closely during the first 48 hrs following the procedure, and after which a standard monitoring time frame will apply. Pain will be managed through the use of analgesics as necessary. Dosing regimens will be explored to reduce the number of injections at different sites. This includes the use of small minipumps which may be implanted under the same anaesthesia as osteotomy surgery, and can deliver agents without the need for repeat injections.

We will also first determine whether the tail vertebrae model is effective in assessing bone regeneration, and where reduced numbers might be used if the more invasive long bone osteotomy model becomes necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow all guidelines indicated by the NVS, NACWO and research institution and including in relevant publications such as 'The Design of Animal Experiments', and the 'Handbook of Laboratory Animal Management and Welfare'.

We will follow the LASA guidelines for aseptic surgery and analgesia, which can be found on http://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf and the Home Office Minimum Standards for Aseptic Surgery (www.procedureswithcare.org.uk/ASMS2012.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend routine Animal Welfare Meetings (former Gold Standard) and continue to engage with Institutional efforts to promote the 3Rs, including our continued participation in Institutional 3Rs day, and workshops.

5. Improving treatment options for individuals with diabetes and developing intervention strategies; using beta cell replacement and regenerative therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Diabetes, Islets of Langerhans, Insulin, Mesenchymal Stromal Cells, Transplantation

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the work carried out under this project is to improve treatment options for people living with diabetes and to develop intervention strategies to delay the onset of diabetes in those at risk. The project will address two main objectives; (1) Improving the survival and function of transplanted islets by reducing early inflammation at the implantation site; (2) Defining strategies that help to prevent chronic immune cell mediated destruction of endogenous pancreatic islets and transplanted islets.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Type 1 Diabetes (T1D) is an autoimmune disease in which the immune system destroys the insulin- producing islet beta cells in the pancreas. Approximately 422 million people worldwide have diabetes, 400,000 in the U.K, and its prevalence is gradually increasing. Diabetes is associated with increased morbidity and early mortality due to the complications associated, including eye damage, cardiovascular complications and kidney disease.

Islet transplantation offers the perfect treatment for T1D offering continuous, real-time, glucose responsive insulin delivery avoiding short-term risks of low blood glucose (hypoglycaemia) and longer term risks of microvascular complications. However, islet transplantation is still an experimental therapy with caveats and clinical limitations. Islet transplantation can improve quality of life for people living with Diabetes who have difficulty managing blood glucose levels despite their best efforts with intensive insulin therapies and extensive advice from diabetes specialists. Following this procedure, the number of daily insulin injections/insulin administration via a pump can be significantly reduced and, in some cases, stopped for at least a few months, and up to five years after. Islet transplantation can improve quality of life by eliminating/reducing hypoglycaemia and stabilizing blood glucose levels, which reduces the psychological stresses associated. Islet transplantation is likely to reduce the probability of long-term secondary vascular complications. Improvements to systemic immunosuppressive regimens has potential to reduce unwanted side effects of some currently used drugs. Improving the efficiency of islet transplantation will benefit individual islet graft recipients, and by saving on the scarce human islet graft material, will allow the treatment of many more people with T1D.

Defining novel therapeutics which improve the functional survival of the insulin-secreting islet beta cells, has significant potential to contribute to improvements in clinical islet transplantation, as a therapy for T1D. Additionally, this work has potential to help maintain the survival of endogenous pancreatic islet beta cells to delay and/or prevent the onset of Diabetes. The strategy to develop therapeutics that reduce inflammation of the transplanted islets themselves, as well as cells within the environment to which the islets are transplanted (immune cells and endothelial cells lining the blood vessels), enhances the likelihood of successful therapeutic intervention.

What outputs do you think you will see at the end of this project?

The studies outlined under this project proposal will generate new information regarding the potential of novel strategies to improve islet transplantation and beta cell replacement therapies for the treatment of diabetes.

The studies will be disseminated through publication in scientific journals and communication of findings at scientific and clinical conferences.

Who or what will benefit from these outputs, and how?

These studies will generate information that will be of direct clinical relevance in improving the outcome of transplantation therapy for Type 1 Diabetes (T1D). At present, clinical human islet transplantation can be offered to only a small subset of people with T1D, who develop severe hypoglycaemia (low blood glucose) unawareness. Unfortunately, at present not all people with hypoglycaemia unawareness can be offered a transplant. Improvements to the efficiency of clinical islet transplantation, as well as immunosuppression strategies, will mean that islet transplantation can be offered as a therapeutic option to many more people with T1D. These studies are also likely to identify potential therapies for delaying the onset or even preventing T1D.

Islet transplantation improves quality of life for patients who have difficulty managing blood glucose despite their best efforts with intensive insulin therapies and extensive advice from diabetes specialists. Following this procedure, the number of daily insulin injections/insulin administration via a pump can be significantly reduced and, in some cases, stopped for at least a few months, and up to five years after. Islet transplantation differs to some of the other forms of treatment such as insulin pumps, because the islets are infused by a simple procedure into the portal vein (liver), which means that the insulin does not need to be delivered by an external technological device ie Insulin pump. Islet transplantation can improve quality of life by eliminating/reducing hypoglycaemia and stabilising blood glucose levels, which reduces the psychological stresses associated. Islet transplantation is likely to reduce the probability of long-term secondary vascular complications. Improvements to immunosuppressive regimens has potential to reduce unwanted side effects of some currently used drugs.

Information generated from this project may impact on the outcomes of islet transplantation within a relatively short period (5 years) by identifying straightforward modifications to clinical islet culture/transplant procedures. Any impact on intervention of T1D onset is likely to take longer (10 years) because of the time frame of drug development and testing in clinical cohorts.

The identification of novel anti-inflammatory/immunomodulatory mediators has potential to improve the survival of stem cell derived beta cells after transplantation. Therefore, in the longer term, this research may contribute to the potential of using alternative islet/beta cell sources as a replacement therapy to treat diabetes. This will further increase the number of people living with diabetes who are likely to benefit from this research.

How will you look to maximise the outputs of this work?

These studies will generate scientific data and information useful to researchers and clinicians within the diabetes field. The information generated from this project will be made available to clinicians in the diabetes field, and members of the UK Islet Transplantation Consortium. The research outcomes will be disseminated at scientific and clinical conferences and in scientific journals.

Species and numbers of animals expected to be used

- Mice: 2300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Aim One and Two of this project is to improve the efficiency of clinical islet transplantation as a therapy for Type 1 Diabetes (T1D). The use of experimental animals is unavoidable, since we need to carry out preclinical studies in mouse models of islet transplantation that mimic the clinical procedure. Wherever possible experiments will be performed in vitro, using islets isolated from mouse pancreas (this is not a licensed procedure under the 1986 Act). However there is no suitable in vitro model for the complex mechanisms regulating whole body glucose control, which involves many tissues and organs, including liver, muscle, fat, endocrine organs and immune cells. We will use adult mice for these experiments, since in the clinical scenario, individuals with T1D who may be offered an islet transplant will be adults.

As part of the scientific objectives of aim two, we will define therapeutic strategies that help to ensure the survival of endogenous insulin secreting islet beta cells that are under attack from the immune system during the development of diabetes. The causes of diabetes are complicated and involve complex interactions between the islet cells and immune cells. Therefore, preclinical studies that can determine the capacity of a therapeutic strategy to delay diabetes onset need to be done in mouse models that mimic the clinical scenario in humans.

Typically, what will be done to an animal used in your project?

For islet transplantation experiments, mice will be administered an injection of a chemical called streptozotocin, which selectively kills the insulin secreting islet beta cells. After this injection mice become diabetic within 5 days. It is necessary that the mice develop high blood glucose, so that it is possible to fully determine the effects that our modification to the transplant procedure has upon blood glucose control and cure rate in islet transplant recipients.

Mice will receive transplants of insulin secreting islet cells, transplanted beneath the kidney capsule, or into the liver via the portal vein. Occasionally, islets may be transplanted beneath the skin (subcutaneous transplantation), into the peritoneal cavity or into the muscle. Mice will be unconscious for the transplantation procedure to ensure no unnecessary stress, and will be administered analgesics prior to the procedure to minimise any pain. Mice regain consciousness following the transplantation and will be monitored in the hours following the transplantation to ensure that they have recovered promptly as

they should do. Transplanted mice are then monitored daily in the first week post transplantation and every 2-3 days for the remainder of the post-transplantation monitoring period.

Mice will typically be monitored (blood glucose and weight) for 4 weeks after transplantation. However, longer monitoring periods (up to 20 weeks) will be necessary in experiments where we need to evaluate the efficacy of a defined immunosuppressive modification to delay immunological graft rejection. Some mice that have been transplanted with islets underneath the kidney capsule may have the islet graft-bearing kidney removed surgically at the end of the post-transplantation monitoring period to check that the cells were working properly. Specifically, mice that have been cured by the islet graft should revert to hyperglycaemia if indeed it is the islets transplanted underneath the kidney capsule that are responsible for the cure from diabetes.

For experiments that aim to determine whether we can delay the onset of diabetes, we will use mice that have the DNA and genetics that mean they spontaneously develop diabetes. In these mice, we will give them defined therapies to try to delay or prevent diabetes occurrence. These therapies will be given by simple procedures, such as injections with defined dosing schedules. The development of diabetes will be monitored by testing blood glucose using a glucose meter (the same way that humans can test) and a small drop of blood collected by making a small pin prick in the tail (this will be the way that we monitor blood glucose in islet transplanted mice too).

What are the expected impacts and/or adverse effects for the animals during your project?Hyperglycaemia (high blood glucose) can cause a small amount of weight loss, which is usually kept to a minimum after islet transplantation due to the blood glucose lowering capacity of the transplanted islets. Animals that have high blood glucose (above 20 mmol/l for 2 consecutive readings) may be given subcutaneous insulin injections to prevent excessive weight loss.

High blood glucose can also cause thirst and more frequent urination than normal. Therefore, availability of water will be monitored regularly and bedding will be changed on a very frequent basis. The insulin injections will also limit how high blood glucose becomes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals used for this project are expected to experience procedures of moderate severity. This includes the mice who spontaneously develop diabetes and the mice that are given an injection to make them diabetic before investigating strategies to improve transplantation outcomes.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the initial studies will use cells and/or islets isolated from experimental animals. These experiments will be performed under controlled conditions outside the body, in vitro, but there is no in vitro model to mimic the complex control of blood glucose following islet transplantation which involves many organs including liver, muscle, fat, gut, lymphoid and endocrine tissues. Experimental in vitro systems cannot fully measure complex interactions of immune cells within tissues. Initial immune cell studies will be done using isolated immune cell subsets in tissue culture. However, understanding integrated immune processes is key to understanding human disease progression and graft rejection.

Which non-animal alternatives did you consider for use in this project?

Detailed in vitro studies, which do not require the use of animals, make up the majority of this project.

We will use immunological assays using immune cell populations from human volunteers, cell lines which are similar to human and mouse cells, and in some cases using cells and tissues collected from animals. These assays will assess whether our therapeutic strategy can reduce unwanted inflammatory and immune attack and destruction of the insulin-secreting islets.

We will also use in vitro endothelial cell lines (the cells which line blood vessels) inflammation assays, to investigate potential therapeutic strategies that may be used to limit inflammation-mediated loss of transplanted islets. Investigating endothelial cell inflammation is important because in the clinical situation, islets are transplanted into the blood vessels of the liver.

These non-animal alternatives are designed to mimic the islet transplantation environment in vitro, so that less animals need to be used to address our scientific questions.

Why were they not suitable?

A number of non-animal alternatives are suitable for investigating the likely effective dose of therapeutics and their mechanisms of action. These will be used before progressing to the animal studies outlined in this project.

However, there are no non-animal alternatives that are appropriate for fully mimicking the complexity of interactions between inflammatory/immune cells, endothelial cells and islets in the setting of Type 1 Diabetes (T1D) pathogenesis or beta cell replacement to treat T1D. Therefore, our promising in vitro studies will need to be investigated using animal models of diabetes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

In general, experiments will use group sizes of between 6-12 animals. Precise group sizes for individual experiments will depend on the scientific question. For example experiments that involve investigating whether a particular treatment has any effect may require small group sizes, whilst an experiment to determine whether there is any significant difference in efficacy between two treatment groups may require more animals, particularly where more variation is expected in the outcomes. For transplantation experiments, animal numbers were calculated based on our own and other previously published studies, which have used mouse models of islet transplantation to determine whether a therapeutic modulation can improve outcomes. The number of mice which have particular genes that make them susceptible to developing Diabetes with a similar progression and underlying pathology to that seen in the human condition, has been estimated based on published studies and communication with colleagues (who have expertise in maintaining these mice colonies) indicating that the incidence of spontaneous diabetes is 10-40% and 70-90% in male and female mice, respectively. It is expected that each mother would have 2, or 3 litters maximum, based upon breeding between ages 6 weeks and 12 weeks, and an anticipated litter size of approximately 5 pups.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design for this project we have referred to NC3Rs guidelines online (https://www.nc3rs.org.uk/experimental-design), to ensure that animal numbers are reduced to a minimum. The outline of this project is designed so that transplantation experiments and intervention strategies to delay or prevent the onset of diabetes will be carried out as an absolute end-point following in vitro experiments that demonstrate robust and reliable efficacy.

Experimental protocols will be designed to reduce animal numbers. For example, in islet transplantation experiments where islets are implanted under the kidney capsule and the

mouse cures from diabetes, the islet graft-bearing kidney will be removed at the end of the monitoring period to assess whether the animal will develop high blood glucose again. This proves that the transplanted islets are the cause of the animal curing, as opposed to regeneration of the islets in the pancreas, and thus removes the need for sham/mock operations in control animals. We will refer to the ASRU GA Breeding and maintenance framework for guidance on breeding and colony maintenance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For experiments using diabetic mice, other tissues, such as the kidney, bladder, brain, heart and eyes may be collected after the mice have been humanely killed, to look at potential effects of secondary complications of diabetes, alongside the primary objectives looking to improve transplantation outcomes as a cure for diabetes or intervention strategies to prevent diabetes.

All in vivo work will be reported according to the updated ARRIVE guidelines, as published this year in PLOS BIOLOGY.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For some experiments, mice will be administered an injection of streptozotocin, which selectively kills the insulin secreting islet beta cells and therefore, the mice become diabetic within 5 days. This can cause a small amount of weight loss. Mice then receive a transplant of insulin producing cells and compounds designed to improve the survival of transplanted cells. This procedure is needed, so that it is possible to fully determine the effects that our modification to the transplant procedure, has upon blood glucose control and cure rate in diabetic animals. Mice will be unconscious for the transplantation procedure to ensure no unnecessary stress and will be administered analgesics prior to the procedure to minimise any postoperative pain. Mice will be monitored in the hours following the transplantation to ensure that they have recovered promptly as they should do. Transplanted mice are then monitored daily in the first week post transplantation and every 2-3 days for the remainder of the 1-month follow-up period. Animals that have extremely high blood glucose will be given subcutaneous insulin injections to prevent excessive weight loss and suffering.

For intervention studies aiming to delay or prevent Diabetes, we will use a strain of mice which has genes that make them susceptible to developing Diabetes. This strain of mice spontaneously develops diabetes in a similar way to humans and so many of the aspects of disease progression and pathology are the same. For example, similar immune cells (T cells) are important in disease progression for humans and this strain of mice, as is the autoimmune (the body's own immune system destroys its own insulin secreting islet beta cells) nature of the disease. This allows us to effectively investigate novel strategies to delay or prevent diabetes.

Why can't you use animals that are less sentient?

All experiments will be conducted in mice, which provide the most ethical model. Mice show similar glucose control mechanisms to humans, so data obtained are valid for translational studies in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal suffering is minimised by aseptic surgical procedures and by postoperative analgesia for all surgical procedures.

Blood samples will be small (less than 1 microliter) and the total taken will not exceed 15% blood volume in any 28 day period. Following sampling, pressure will be applied to the puncture site to stop bleeding. When taking blood samples mice will not be restrained as this will cause unnecessary distress. Instead, they will be allowed to move around the cage freely, whilst taking the blood sample by a small pin prick to the end of the tail, which can be done in a matter of seconds.

Fasting of mice to achieve baseline blood glucose prior to glucose tolerance tests will be limited to a minimum to reduce distress where possible. Mice will always have free access to water.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidelines available on online resources including NC3Rs (https://www.nc3rs.org.uk/), Frame https://frame.org.uk/ and the "Strategic Planning for Research Planning Programmes" guidance available here, articles on pubmed (https://www.ncbi.nlm.nih.gov/) and LASA guidelines (https://www.lasa.co.uk/) will be followed, and reviewed on a regular basis.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Scientific literature regarding the most appropriate animal models of diabetes (Pubmed) will be reviewed regularly. The NCR3Rs website will be reviewed regularly

(https://www.nc3rs.org.uk/) to determine where there are potential refinements to procedures, as well as opportunities to reduce and replace the use of animals. I will further discuss possible refinements with the establishment named vetinary surgeon.

6. Modelling cancer biology and therapy in mouse

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph

Key words

cancer, cancer stem cells, therapy, metastasis, tumour

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify genes that influence the development and progression of cancer and to demonstrate whether alteration of these genes (or gene products), either directly or through pharmacological intervention, has a therapeutic effect.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The spread of tumour cells throughout the body is the cause of more than 90% of patient deaths and there is currently a dearth of available therapies for this clinically important, but poorly understood process. Our work aims to identify genes that influence this process and to test novel therapeutics and therapeutic approaches that interfere in these genetic pathways, thus preventing the development and progression of cancer within the body.

What outputs do you think you will see at the end of this project?

New information on the underlying biology of cancer that drives the development and spread of the disease around the body. This will be disseminated through publication of our findings in academic research journals and will lead to new grant funding to explore these findings further. We aim to identify new therapeutic agents that target these novel biological processes and this may lead to the development (and publication) of novel therapeutic products for use in future clinical trials.

Who or what will benefit from these outputs, and how?

There will be the immediate impact of our work to the cancer research community with respect to the addition of new knowledge of cancer biology.

Regarding its long-term impact on human health, the subsequent development of therapeutic agents identified during this project, to the point where they may be used in clinical trials of cancer patients, is a long-winded process requiring further extensive testing of the toxicity and efficacy of these agents in animal and laboratory models. These subsequent tests lie outside the remit of this project.

Consequently, the introduction of any novel therapeutic agents identified during this project into clinical trials and its subsequent impact on cancer patients would take a minimum of 2-3 years after the completion of these studies.

How will you look to maximise the outputs of this work?

All knowledge generated during this project will be disseminated as peer-reviewed publications and presented at national/international conferences/scientific meetings. Data will be shared via open access journals and repositories. We will continue to actively engage with the general public via events within the University/local communities and via national cancer charities. Progress and successes in key milestones will be released via University research pages, newsletters and social media.

Species and numbers of animals expected to be used

- Mice: 2250 adult males and females

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The progression of cancer, where tumours grow then disseminate to other parts of the body, is a complex multi-stage process that encompasses many changes in the interactions between tumour cells and a large variety of different 'host' tissues. These interactions are essential for the success of the tumour to spread, and each has been modelled in the laboratory to some degree - but in order to represent the entirety of this process - and thus to model the effects of inhibiting one or more of these elements on clinical outcome, it is necessary to observe the process in a complete animal model.

We intend to explore the genetic basis of this process and mouse transgenic models are the most advanced genetically tractable models which approximate most closely to the tissue architecture of humans. They also remain the primary model for the propagation of human tumours within a whole organism host in which human tumours retain their tissue architecture and ability to disseminate and seed at distal sites within the host.

Typically, what will be done to an animal used in your project?

Mice will either be genetically predisposed to developing cancer or will be transplanted with cancer cells and then the effects of anti-cancer agents or alteration of the genes of interest will be assessed.

In the case of genetic predisposition, existing genetically altered mice will be sourced from other projects with authority to breed and maintain genetically altered animals, and subsequently maintained and bred to generate experimental mice with predisposition to develop tumours.

Animals with genetic predisposition will be monitored or scanned for signs of tumour burden and may be subjected to administration of substances or cells that may affect the course of tumour development (via one of several routes including for example, blood stream, subcutaneous or intra-muscular).

In the case of transplantations, either wild-type or genetically altered mice will be injected with cancer cells (under the skin, within the body cavity or organ or into the bloodstream) or surgically transplanted with tumour tissue and monitored or scanned for signs of tumour burden and may be subjected to administration of substances or cells that may affect the course of tumour development (via one of several routes including for example blood stream, subcutaneous or intra-muscular).

What are the expected impacts and/or adverse effects for the animals during your project?

The primary adverse effects originate from tumour burden. These include adverse effects of malignant tumours such as weight loss, hunching, inappetance, lack of grooming. Additional tissue-specific clinical signs of pancreas tumours may include weight loss from the haunches, abdominal distension, pallor in the extremities (e.g., paling feet). Lung

tumours may cause breathing difficulties. These signs of morbidity may be evident for several days but usually no more than one week before humane killing. In some models of disease spread (metastasis) there may be infrequent (approximately 5%) instances of sporadic sudden death brought about by vascular blockages caused by the circulating tumours cells.

Following surgery (during embryo transfers or transplantations) there may be tissue inflammation which may cause transient minor discomfort and will be managed with the use of analgesics.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 40% of mice will either harbour no genetic alteration as a result of breeding or will be harbouring a genetic alteration (either immune compromised or harbouring a genetic alteration potentially affecting tumour burden) but will be symptom free and therefore will be categorised as Mild.

Approximately 60% of mice will either have developed tumour burden spontaneously due to their genetic alteration or will have been transplanted with tumour cells and their symptoms will have been managed with respect to the adverse effects of tumour burden, so will be categorised as Moderate.

Approximately 1% of mice may experience sudden onset death (as a result of metastatic tumour burden) and will be categorised as Severe.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to be able to model the complex biology of tumour interactions with normal cells in a tissue microenvironment in which the tumour cells are able to migrate from the proximal site to distant tissues via the lymphatic and/or blood system. This allows us to understand the role of individual (abnormal) genes in complex tissue systems and also allows us to determine the effect of disrupting these genetic pathways on the metastatic process.

Mouse models of cancer are clinically relevant and accurately recapitulate the full spectra of human disease at the genetic, cellular and tissue level. Cancer is multifaceted disease that initiates with genetic mutation in cells; however, for tumours to progress to malignancy mutant cells 'hijack' normal biological processes (e.g., immune cell infiltrate, stromal cells, extracellular matrix modification) to support tumour progression. Mouse models accurately model all aspects of tumour complexity that would be technically challenging to achieve using ex vivo or in vitro cell systems alone.

Which non-animal alternatives did you consider for use in this project?

We use a variety of non-animal alternatives in our research. The majority of these involve monolayer cell culture, 3D-organoid and primary tissue (human and mouse) culture in the laboratory as a reductive approach to unravel molecular cell biology of a phenomenon at the single cell level. Organoids also allow for high-throughput experimental approaches, e.g., testing efficacy of pharmacological inhibitor before administering to animals. However, these systems cannot entirely replace the need for animals in this project.

Why were they not suitable?

These alternative models and assays each measure single aspects of cancer progression: for example, cell migration, cell division, cell death and there are numerous variations in practice that include mixed cell types (tumour and non-tumour) in the assays. However, none are capable of recapitulating the series of events leading to cancer metastasis, or mimic the multiple cell interactions that it involves.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is estimated empirically to be 75% of number of mice used in the preceding project licence for this project. This takes into account the increased use of alternative non-animal models used in our work and the evolution of the project towards pre-existing animal models which minimises breeding requirements.

We estimate this will include 3 genetically altered mouse lines involving multiple conditional oncogenes and experimental transgenes. Transplantation experiments will be performed according to our published studies and we predict that the number of experiments to be performed will be approximately two-thirds the number performed under our previous project licence as we will be completing final preclinical evaluation of Bcl3 in

Objective 2, while progressing cFLIP studies (Objective 1) and up to two additional target gene pathways from Objective 3.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

There has been an increased use of non-animal models of cancer in our laboratory and previous introduction of scanning modalities which allows us to non-invasively monitor tumour progression in mice over time rather than have to use multiple animals at single time points for specific experiments investigating disease progression.

All experiments are based on previous studies performed within the host laboratory using identical or similar mouse models designed using the NC3Rs Experimental Design Assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to source experimental animals for transplantation studies from other projects or establishments with authority to supply genetically altered animals (eg commercial services) rather than breed our own stocks which can lead to the generation of 'excess' stock animals.

In the majority of cases optimum cohort sizes for individual experiments have already been established through empirical observation from our previous licensed work or from the published literature. In each case the minimum number of animals required to obtain statistically rigorous outcomes have been identified. Specific measures that have been incorporated into our experimental designs to reduce mouse numbers include: breeding heterozygous pairs/trios to increase efficiency in the generation of transgene heterozygosity; dividing harvested tumour target tissues into multiple fixatives (e.g., OCT, FFPE, frozen) in order to maximise the output of each induced animal; including pilot studies (2-4 mice) to ascertain optimal dosing of pharmacological inhibitors

Transplantation of established tumour lines dramatically reduces the variability of tumour latency and growth kinetics compared to primary tumour transplants and sporadic tumours. Power calculations have already been applied to many of these transplant models and, due to their inherent uniformity, relatively small tumour cohorts (eg. n>6) are required for statistical rigor.

Where possible, bilateral transplants will be performed in order to halve the number of animals required to generate sufficient experimental tumours. This may include asymmetric bilateral transplants in which experimental tumour cells may be transplanted opposite to an unmodified (control) tumour in the same mouse. This has the advantage of minimizing variability between experimental and control arms of an experiment, thus reducing cohort sizes

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Breeding strategies and long-term tumour cohort studies require that the animals be maintained for prolonged periods of time (more than one year). In all cases, but particularly for these animals, care is taken to ensure where possible that they are kept in small sibling groups (more than 2) in appropriately sized cages and that suitable 'apparatus' is provided to help enrich their environment.

The principal impact on animal suffering in this project will be tumour burden and the involvement of secondary tumours to vital organs. This may occur in GA mice or following tumour transplantation. Suffering will be minimised by: (i) close adherence to maximum permissible tumour size according to Workman and co-workers (Workman P., Aboagye, E., Balkwill, F. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010). https://doi.org/10.1038/sj.bjc.6605642); (ii) using, where possible, established GA models and established transplantation models, with predetermined tumour latency, growth kinetics and secondary tumour involvement; (iii) use of non-invasive imaging techniques to identify secondary tumours early, prior to external signs of morbidity; (iv) use of tissue specific and inducible conditional transgenics in GA mice to restrict aberrant phenotypes and tumour growth to adult tissue.

Suffering may also occur following adverse reaction to transplantation, surgery or injection. We have pro-actively refined our intravenous and subcutaneous xenograft techniques to significantly improve recovery rates. For example, intravenous injection of tumour cells is now performed under light or no anaesthesia to alleviate pulmonary distress and significantly improve recovery rates.

Why can't you use animals that are less sentient?

The most appropriate tumour models must recapitulate the tissue and circulatory architecture of humans and so mammals (which possess mammary structures to mimic breast cancer for example) and higher vertebrates (pancreas, prostate, upper and lower GI) are the most appropriate experimental models for the majority of solid tumour types in humans.

Tumour development and progression is primarily a disease of adult/aged tissues and occurs over a period of weeks or months and so it is necessary to perform these

experiments in adult animals for prolonged periods of time. Therefore, immature life stages are not appropriate for our research studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements will include frequent monitoring of induced animals, post-operative or anaesthetised animals. Exogenous substances/agents will be administered at the minimum dose, as determined using NC3Rs guidelines for best practice and dosing regimes, and following consultation with our collaborators. All procedures will be carried out by trained and experienced staff with the support of experienced animal facility staff/NACWOs/NVS (e.g., application of anaesthesia, analgesics, surgical techniques). Clear humane end points are detailed for each protocol.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

This project will be carried out in accordance with ARRIVE guidelines and involving collaboration with experienced cancer researchers with track records of excellence in the field of tumour xenografts and in the use of genetically altered mouse models of disease.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Engage with our Establishment’s Regional Programme Manager for the NC3Rs on a regular basis via local workshops/meetings, newsletters and social media.

7. Modelling brain tumours in the mouse

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

brain tumours, cancer stem cells, neural stem cells, radiotherapy, epigenetics

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Brain tumours are the second leading cause of cancer related deaths in males ages 20-39 and the fifth leading cause of cancer-related deaths in women ages 20-39. Patients with glioblastoma survive about 140 days on average rising to 14 months if they are suitable for radical treatment. Survival trends for these patients have not improved in recent years and these tumours represent a rare exception to the general trend of cancer survival in the UK. Brain tumours also account for a large proportion of childhood tumours. Medulloblastoma is the commonest brain cancer seen in young children. The main treatments available to children with medulloblastoma today are surgery, radiotherapy or chemotherapy. These treatments can be effective and kill the tumour cells in a proportion of patients; however they are almost invariably also causing severe side effects which are particularly damaging in young children as their brain is growing quickly.

We are studying the cell of origin of brain tumours and we are trying to identify novel genes and pathways that control their behaviour. In particular we are looking at stem cells:

these cells are found in every organ and can develop into specialised cells, thus contributing to the maintenance and 'regeneration' of the organ. We find cells behaving as stem cells in tumours too, they play an essential role in "regenerating" the tumours and so killing them is crucial. We are trying to establish the difference between these tumour stem cells that keep proliferating – producing other tumour cells endlessly – and normal stem cells, which stop proliferating when the body stops needing new cells. If we can understand how a tumour stem cell controls its proliferation, we can start generating more targeted drugs that specifically kill these cells in a more effective and also much less toxic way.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This research will help us to understand how to improve the treatment and therefore the survival of patients with brain tumours. If we know what allows tumour to grow, we will be able to design new more effective therapies.

What outputs do you think you will see at the end of this project?

The research data to be generated are:

- generation and characterisation of advanced pre-clinical models of brain tumours
- description of mouse brain tissue as well as establishment and characterisation of mouse cells cultured in a dish, both healthy and in the context of tumour development
- whole genome datasets obtained through RNASeq, small RNASeq, ATACSeq, ChIP Seq and WGS, on mouse tissues and cells
- design and validation at pre-clinical level of novel therapeutic approaches, including testing 10-15 compounds with the aim to take the most promising forward to clinical trials.

We will disseminate the results and conclusions of the research to broader audiences. We will present the work at meetings and at invited seminars at various institutions. We will publish our findings in international peer-reviewed journals with the best possible visibility. We will organise and participate in workshops, charity events, and road shows to provide training and dissemination of information. Our lab has a close relationship with several charities and we host regular lab visits, which include direct involvement of patients and their families in the design of our studies. We regularly interact with clinical patient groups in the context of grant applications (preparation and review).

Who or what will benefit from these outputs, and how?

This basic science programme of work will provide us with a better understanding of the molecular basis of the development of malignant brain tumours, in particular it will elucidate the contribution of molecular changes to DNA structure impacting gene activity (epigenetic changes) to the regulation of tumour formation and maintenance.

This project will have important implications not only for cancer research and therapy but also for neuroscience, stem cell biology and developmental biology.

Most importantly, this project has the potential to significantly impact on patients' health. Gaining a better understanding of the underlying biology and development of brain tumours, is essential for drug development. The work undertaken as part of this project uses, wherever possible, cells directly derived from human brain tumours, and uses the most up-to-date in vivo models in which to test novel and repurposed treatments. The brain tumours we study have some of the worst prognoses of any cancers, and work such as that which is proposed here is essential to find treatment pathways that will significantly impact on patient survival outcomes. This is only made possible by using experimental systems that best recapitulate brain tumours by using environments as close as possible to human disease. One of the overarching aims of this project is to provide robust pre-clinical evidence that could inform the design of future clinical trials.

We will work with science journalists to submit press releases of our most prominent publications to reach the goal of engaging a wide public audience. Moreover, our research will provide new contents for the Institute and School website.

We will engage with charities, patient groups and all parties' parliamentary groups to enhance public understanding and awareness of our research.

How will you look to maximise the outputs of this work?

We have an extensive network of collaborations both nationally and internationally. In generating data that can be accessed on open forums and presenting at national and international conferences, we will ensure that the wider research community is able to benefit from our work.

This programme of work promotes collaborations between wet-lab scientists and bioinformaticians, providing training that bridges different research fields. Indeed recent developments in high-throughput methods place significant challenges to next generation life scientists and clinicians, who are expected to have expertise in molecular and cellular methods but increasingly require also in depth knowledge of bioinformatics approaches to be able to develop interdisciplinary programs. Individuals involved with the project will directly benefit from the interdisciplinary training provided and transferable skills obtained.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have many physiological and genetic similarities to humans, therefore they represent excellent models for studying complex diseases and response to treatments that affect humans. Additionally, the lifespan and reproductive process in the mouse allow experiments modelling whole disease courses to be carried out on a relatively short timescale. There are extensive tools available, in our lab and beyond, for the manipulation of the mouse genome also making the mouse an excellent model, and especially important for cancer research, immunodeficient mice are available that allow human cancers to grow within a mouse - allowing detailed research into how cancer interacts with the host environment and the best model for new treatments.

We will use both young and adult mice, as we want to use mice of ages that match those in which human disease occurs. Glioblastoma and metastatic brain tumours are the most common brain tumours that occurs in adults, and medulloblastoma is the most common brain tumour of children.

Typically, what will be done to an animal used in your project?

The majority of mice used in this project will be used for breeding and these mice will only undergo momentary discomfort for marking procedures. A proportion of mice will undergo induction of brain pathology, which will entail either genetic induction of tumours or injection of tumour cells, either directly or via the bloodstream, into the brain. Subsequently a proportion of these mice will undergo treatment for the resultant tumours with drugs and/or radiation and/or simulated biopsy. Mice typically take from two weeks to a few months to develop tumours, and will be humanely culled once symptoms develop or at defined timepoints after induction of brain tumours to study their origin and development. Tumour cells may be labelled to facilitate their identification within the brain and their isolation for analysis.

Some mice will undergo focal irradiation to their brains, for the purpose of examining the molecular mechanisms that underly the side effects of radiotherapy. Some of these mice will be given drugs to test whether they can be used to protect against these side effects.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of the animals will develop sign and symptoms related to the induced brain pathology including circling, holding the head on one side, disturbance of equilibrium, reduced movements, hunchback position, hypersensitivity upon touching and handling, decrease in food intake, separation from cage mates, disturbance of eye movements, limb weakness. Rarely they may also exhibit no activity when stimulated, persistent laboured breathing, difficulty in locomotion restricting access to food or water, seizures, or limb paralysis. The duration of these effects is usually short (hours-days), and mice will be humanely culled once symptoms develop as per established protocols.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We will use approximately 12000 wild type and genetically modified mice over 5 years. Whilst a proportion of these mice will develop symptoms of the diseases we want to study, the majority of them will only be used for breeding and/or for procedures which do not cause discomfort to the animals. The overall severity of these experiments is moderate, we expect 40% of the total number of mice to fall in the moderate category.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Complex diseases such as brain development and tumour formation can only be studied in whole organisms and the mouse is a well characterized model system to mimic human diseases. Mice are the animals best suitable for this type of experiment: They are vertebrates and they are mammals, so they share many of the physiological properties found in humans. The mouse, like humans, develops similar brain and the presence of stem cells within it as well as the almost identical mechanisms involved in growth and differentiation of these cells makes the mouse brain ideal model systems to study diseases of this organ. Moreover, the mouse is the only mammal in which the genetic tools to specifically delete genes in specific cell types are available. The of new mouse models, closely mimicking the human neoplasms, are very valuable tools to evaluate response to new therapeutic approaches.

Which non-animal alternatives did you consider for use in this project?

For many mechanistic studies, in particular the cooperation of genes, we will use, whenever possible, cell biology and molecular biology methods, mainly primary cells but also cell lines. For example, in vitro models have been developed in which cultured neural stem cells and progenitor cells as well as primary tumour cells derived from surplus surgical material are used. With this approach we plan to gain insights into control of proliferation and differentiation of normal and neoplastic cells lacking or overexpressing specific genes. Importantly we have introduced 3D organoid-based in vitro model to mimic some aspect of tumour biology such as the interactions between the tumour cells and the surrounding brain tissue.

Why were they not suitable?

To establish whether a tumour originate from a stem cell in situ and how tumour cells migrate through the brain and interact with other resident brain cells, such as for example inflammatory cells or blood vessels, cannot be simulated in vitro, not even in the context of 3D organoids. Likewise preclinical validation of novel treatments, including testing efficacy and side effects, can be achieved only in a in vivo setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We are constantly reviewing our procedures in order to improve wellbeing and to reduce the numbers of animals used. Where possible, we utilise approaches that do not require animals such as cell culture and computational biology. Unfortunately, it is not possible at the moment to completely replace the use of animals with other approaches.

We plan to work with approximately 30 different genetically modified mouse strains (already available or newly generated according to the needs of the project) over the 5 years of the project licence. We have many years of experience in using the mouse as a model system and experiments are designed to use the lowest number of animals required to generate statistically significant data. All the strains will be bred for maintenance (Protocol 1) and for supplying mice to experimental protocols (Protocol 2, 3 and 4). According to our previous experience approximately 350 mice per strain per year will be necessary. This number includes animals that will be unsuitable for further experimental use, which is inevitable when generating compound mutants with complex combination of genotypes. This is due to the fact that some experiments require very specific genetics and to establish the breeding of mice with a very specific genotype, several rounds of

breeding are required, and in each of these rounds only the mice with the correct genotype are selected, whilst those that do not inherit the mutations of interest cannot be further utilised.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

NC3R guidelines have been used, and animal group sizes (usually 8-10 animals for experimental groups) have been set after consultation with a statistician, and with consideration of our own previous xperimental work. The relatively high number of animals in each experimental group is due to a certain degree of variability of the parameters (cell growth, cell division, brain invasion or cellular death, etc) that are going to be analysed. All our experiments are planned on the basis of statistically robust sample size calculations to ensure the experimental groups are neither too large nor too small to detect the effects of the procedures. Control groups will be shared between studies whenever possible Occasionally historic controls may also be used, if appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, lines will be bred to homozygosity to increase the frequency of useful experimental animals, thereby reducing the total number of animals needed.

Increase of survival is the most commonly used approach to determine treatment response. However, as this determines an endpoint only and cannot capture the dynamic of tumour growth and regression we will monitor a representative group of xenotransplanted mice by in vivo imaging, which can determine tumour volume, prior to endpoint. This reduces the number of mice, in that timepoint culls are not necessary to assess growth dynamics of a tumour.

Moreover, when possible, alternative approaches will be used to minimise the number of mice. For example, intracranial injection of viruses will be used to initiate tumours in the brain when appropriate (Protocol 3). With this method the crossing of genetically altered mice strains is not needed and therefore allows the reduction of the number of mice used in these experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically engineered mouse models, viral-mediated delivery of tumour inducing genes and orthotopic xenografts of human brain tumour cells. Surgical procedures will be performed under conditions of strict asepsis, surgery will not be commenced until areflexia, as sign of appropriate depth of anaesthesia, is demonstrated. Circulatory and respiratory monitoring will also be carried out to confirm depth of anaesthesia. Analgesia will be used as appropriate to alleviate pain and distress and all animals will be kept warm during and after surgery and inspected regularly. We have over the last few years significantly refined the method of anaesthesia and the method of injecting cells and reagents. This has resulted in a very small number of unexpected complications and faster recovery from anaesthesia.

Why can’t you use animals that are less sentient?

Non-mammalian animals have many significant limitations as models for the study of the initiation and development of human brain tumours: they do not share as many of physiological and stem cell properties with humans as mammalian models, and the genetic tools available for their study are more limited. The choice of life stage of the animals to be used has been made to ensure we best mimic human diseases – all studies relating to glioblastoma and brain metastasis biology will be performed in adult mice, whilst all studies examining medulloblastoma will be performed in un-weaned mice (induction of tumour) and juvenile or adult mice (treatment). The development and growth of a tumour as well as whether a novel treatment is effective can only be studied in living mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals that are expected to develop pathological phenotypes, including developmental abnormalities, neurodegeneration or tumour growth will be closely monitored for pre-defined humane endpoints specific to each phenotype, and culled as soon as these endpoints are met. This will ensure that the suffering of animals is kept as brief as possible and to the minimum possible severity. We have extensive past experience with these phenotypes, all humane endpoints are specifically tailored to monitor brain pathology and are in agreement with the UKCCCR guidelines on humane endpoints and limiting clinical signs. Brain imaging will be used where possible to develop scientific endpoints that allow animals to be killed before symptoms of pathology develop.

All surgical procedures will be performed aseptically, with the most reliable and least stressful analgesia and anaesthesia used for each procedure. Animals will be cared for in the post-operative period, including close monitoring, maintenance of body temperature with heat mats, and provision of fluid support and palatable food wherever necessary.

Two non-schedule 1 methods will be used in selected cases. Animals of all ages (from newborn to adult animals) will be killed by decapitation with prior anaesthesia when isolation of primary neural (neuronal and glial) cell cultures or of primary tumour cell cultures is planned. Perfusion fixation under terminal anaesthesia results in a better conservation of tissue architecture in certain organs including brain, nerves and muscles and improves the quality of subsequent tissue analysis (for example cryosectioning and immunofluorescence, vibratome sections).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All the experiments planned for this research build on a long tradition at this institution of treating animal welfare as a priority. Almost all of the experimental approaches to be adopted have been performed here many times in the past and are associated with detailed standard operating procedures aimed at minimising the number of mice to be used. When any new genetic lines of mice are generated, or if a substance is administered to an animal, they are examined very carefully for any signs of abnormality.

We perform extensive literature searches to assess the continued validity of our models and to design,develop and implement measures which refine lesioning methods particularly in relation to lesion size and severity. We use anaesthesia and analgesia when necessary. We have recently introduced multimodality imaging, which allows examination of animals at multiple and repeated timepoints, importantly also at times when the animals are asymptomatic. This enables a timely identification of the lesions and also a reduction of the number of animals required.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs by regularly checking the resources available on the NC3Rs website (https://www.nc3rs.org.uk/the-3rs) and subscribing to the NC3Rs mailing list for regular updates. We will implement any advances that we can over the course of the project in close cooperation with the named veterinary surgeon.

8. Mechanisms underpinning thrombus formation, stability and vessel repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

thrombosis, stroke, heart disease, antithrombotic drugs, platelets

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this research is to understand the normal and disease processes that govern thrombus formation and breakdown. In the future this will guide on the design of new more effective drugs to prevent thrombosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Thrombosis kills 1 in 4 adults in the Western World. Current drugs on the market are only effective in a subset of patients with many experiencing further episodes of blood clot

formation. A major concern is the side effects associated with these drugs, in particular bleeding complications. Understanding the body's own 'control' system against unwanted clot formation could allow us to target thrombi in disease situation more effectively. To do this we need a more precise understanding of all the different elements that regulate these pathways in vivo. This will enable us to identify mechanisms that may be more effective and safer targets for new antithrombotic medicines.

What outputs do you think you will see at the end of this project?

This translational project will generate new information on the mechanisms that underpin thrombus formation and stability in vivo. The outputs will be disseminated at conferences at both the national and international level and will be published in scientific journals.

Who or what will benefit from these outputs, and how?

Thrombosis is a major issue that affects 1 in 4 people worldwide. Heart and circulatory diseases cause more than a quarter (27%) of all deaths in the UK, equivalent to more than 160,000 deaths each year or one death every three minutes. Healthcare costs relating to heart and circulatory diseases are estimated at £9 billion each year. The cost to the UK economy (including premature death, disability and informal costs) is estimated to reach £19 billion each year. While we have successfully reduced the number of deaths associated with thrombosis in the past 20 years there is no change in the prevelence of the disease. In fact, with an ageing population and escalating obesity, metabolic syndrome and diabetes in the Western world the rates of thrombosis are increasing. We need to develop more efficient ways of diagnosing and treating thrombotic complications to improve patient care and reduce the burden on society.

Our research will focus on defining the mechanisms governing thrombosis & haemostasis. A deeper more developed understanding of these pathways and interactions will permit the design of more specific and tailored treatments in the future. The information gained from this project will likely benefit patients within 10 years as below:-

Short term: colleagues and collaborators will benefit from the data directly and inform on further research.

Medium term: novel methodology and tools, such as biosensors

Long term: drug development/pharma benefits if potential novel therapeutic targets are identified.

How will you look to maximise the outputs of this work?

We have several collaborators around the UK and of course the funders will also disseminate the outputs from the project. Publications arising from this research will be communicated within the scientific community but also to an audience more widely such as conferences to scientific and clinical communities. In addition, we hold several outreach

activities to publicise our research on blood clots in a community setting, including pupils at various stages of education.

Species and numbers of animals expected to be used

- Mice: 4800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The adult mouse model is relevant to our studies because they are the lowest sentient species in which we can address our questions relating to the mechanisms governing thrombosis and haemostasis. The mouse is the most widely used and accepted animal system for these types of studies. There are also an extensive array of genetically manipulated mouse models and established techniques available.

Typically, what will be done to an animal used in your project?

Blood will be harvested under anaesthesia. The procedure will be performed once per animal under anaesthesia after which the animal will be humanely killed.

Other mice may be born with a mild genetic defect that does not cause distress or pain. Mice may be mated with other mice carrying the same genetic modifications. Other mice will have blood collected under anaesthesia and then will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

- Genetically altered (GA) animals will be maintained with no significant impairement of their well- being. The colony will be carefully maintained and monitored by the animal care staff who have experience of changing phenotypes, breeding and birthing problems, changes to pre weaning loss etc

- Animals will be fully anesthetised by experienced staff using an appropriate method of anaesthesia. The appropriate depth of anaesthesia will be maintained throughout the procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity level for this protocol for all mice is mild (GA animals) (57%) and non-recovery (43%) blood collection will be performed under terminal anaesthesia.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The majority of our work (80%) is carried out ex vivo using human blood from normal volunteers or patients samples. The use of animals is currently still required due to the complex nature of the haemostatic cascade. Multiple factors govern the regulation of this system, including circulating proteins, various blood cells, flow rates and the vascular endothelium. It is necessary to evaluate the impact of these components simultaneously to understand how this system is regulated. We also require blood from genetically modified animals which lack specific proteins of interest. Establishing the importance of these proteins is only feasible by genetic manipulation which must be achieved in an animal system.

Which non-animal alternatives did you consider for use in this project?

We considered and will use human subjects to address some of the questions in this project.

Why were they not suitable?

Experiments with human subjects are limited to ex vivo whole blood models, due to ethical reasons. We have various models we can utilise to mimic the human vasculature, which include flow and blood cells. However, these systems lack the endothelial surface and therefore important regulators of the haemostatic cascade. We are also unable to knock out specific proteins to directly assess their function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The major use of animals is in the generation of genetically altered mice and their litter-matched controls. The experimental design involves undertaking a series of studies to investigate cellular and functional processes governing thrombus formation and stability. The majority of these studies will give clear-cut answers from between 5 – 8 animals, although in some situations, we may need to increase this to obtain significance or to obtain sufficient sample. For example, where tests are known to be associated with wide variation such as flow-based studies of thrombus formation or where we require to isolate sufficient cell numbers for flow cytometry analysis. We will use information from our collaborators with experience of these specific mouse lines, including litter size and obtain advice from the animal care staff to ensure that we apply an optimum breeding strategy and minimise the number of breeding animals required. These strains are not currently being used by other laboratories in the UK and therefore it is necessary for us to set up these breeding colonies. Unfortunately, it is not feasible to ship blood from our collaborators in the USA, as the cells are unstable, will become activated during transit and have a short life-span. We will maximise the use of additional mice by using the wild-type mice breed as controls in the experiments. Heterozygote mice will be used to optimise and refine experimental protocols to overall reduce the numbers required.

For studies to investigate haemostasis and thrombosis in mice we will use established statistical tests to determine the statistical significance of a result. Where appropriate, we will consult with a statistician to ensure that we are using the appropriate number of mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Reduction will be achieved by first performing in vitro experiments on human blood to optimise techniques, such as the ex vivo model of thrombus formation. Preliminary work on these mice strains was performed in a collaborating laboratory. Combined with published work this has provided us with information in terms of designing our experiments and the number of animals required to perform such assays. We maximise the number of tissues we can obtain from a single animal and can store blood cells and products and progenitor cells from the bone marrow and spleen for later biochemical studies and other tissues for immunohistochemical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animal numbers bred for use on this Project will be minimised as far as possible by matching breeding to experimental requirements. Pilot studies, appropriate statistical analysis and power calculations will be employed to refine the number of animals used.

The methods chosen will generate the greatest amount of data for the fewest animals used. To maximise the information gained from a single animal we aim to take perform multiple in vitro analyses on each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse haemostatic system is considered a strong model for that found in humans and has the advantage of genetic manipulation. Therefore, in this project we propose to use mice to address specific aims of the proposal that can only be addressed by genetic ablation of specific haemostatic proteins and receptors. Pain, suffering and distress will be minimised as we aim to perform these procedures on mouse blood which will be collected following terminal anaesthesia.

Genetically altered mice strains. The animal care staff will closely monitor the colonies and are experienced in the detection and analysis of phenotypic changes, health concerns and other colony changes including birthing problems, pre weaning loss etc. Any animals showing signs health concerns will be used for tissue or humanely killed. The unit uses GA passports and phenotypical abnormalities will be recorded and if animals are sent to a collaborating institute this information will be released with them.

All mice will tube handled or cupped, no mice will be single housed other than for welfare reasons (e.g. fighting).

Why can’t you use animals that are less sentient?

Non-sentient animals cannot be used for our studies since they lack recognisable platelets and other essential components of the haemostatic cascade.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are committed to refine procedures in line with the 3Rs and will explore various mechanisms to minimise welfare costs. To ensure this:

1. Members of the laboratory will be encouraged to check the NC3Rs resources page

2. Our Named Information Officer will keep us up to date regarding changes in best practice, new courses and will advise of additional training that is necessary to comply with changes in legislation.

3. The laboratory is continually monitoring scientific literature in the haemostasis field, where refinements to these types of procedures are reported. If there are significant changes or advances in practice will up-skill either through training courses or visiting laboratories to receive guidance.

4. Advice will be taken from the experienced animal care staff to ensure optimal procedures and being utilised and incorporated into the daily care of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance for the project will be taken from the NC3Rs website (hubs and microsites). This includes links to publications, other online resources, and video and training materials. The vast array of resources guide on the general principles underlying the experiments highlighted in this project, including anaesthesia, breeding strategy and numbers, and experimental design. Changes in current guidelines will be implemented to the project in line with current literature and published reports. We consult with experts to implement improvements in animal welfare. Local refinements will also be implemented.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are registered to the NC3Rs and subscribe to CRACK IT innovation platform and receive updates on advances through NC3Rs newsletters, and various websites including Norecopa, FRAME and Centre for Alternatives To Animal Testing (CAAT).

9. Developing therapeutic drugs for cancer including the establishment of tumour models

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph

Key words

cancer, therapy, drug discovery, pharmacology, tumour models

Animal types	Life stages
Mice	adult, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop new and improved medicines for the treatment of cancer. As part of this aim, we also plan to develop new cancer models to try to improve the relevance of our animal studies to human disease, or to refine existing models.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In 2018, over 17 million new cases of cancer were diagnosed worldwide, while 9.6 million people died from the disease. It is estimated that by 2040 there will be 27.5 million new cases of cancer each year. In the UK, over a quarter of all deaths are cancer-related. Cancer is a complex disease with more than 200 types of cancer described and the most prevalent types are lung, colorectal, breast and prostate. If cancer is untreated, in the majority of cases it will lead to death. (Source: www.cancerresearchuk.org)

Despite recent advancements in the treatment of cancer, there are still many types of cancer for which therapies are not available. In other cases, therapies are available but they are effective for only a limited percentage of patients. In addition, many patients who do respond to existing therapies eventually develop resistance to these treatments.

Our research project aims to use animals to support our efforts to discover and develop novel therapies for cancer. The use of animals will enable us to mimic complex aspects of the disease in a way that is not possible using cells grown in a laboratory setting. By doing so, we can test the activity of our new drug candidates on tumour growth in a more relevant way in a live animal. The use of animals in our research enables us to develop new cancer drugs to treat patients who have terminal disease and few treatment options.

What outputs do you think you will see at the end of this project?

This work will provide important data to advance our experimental cancer drugs through the phases of research and development by evaluating their activity in animal models of disease. It will further help us to identify the most appropriate drugs to combine together and will guide clinical teams on dose levels and dosing schedules.

This work will also allow us to understand how our drugs distribute in the body after dosing, and how quickly they are eliminated from it.

This project will also increase our insight into how our drug candidates work and will help discover specific changes in the body that happen in response to the drug. These changes can then be measured in human clinical trials.

This work will further enable us to identify potential targets for new drugs in order to expand treatment options for cancer patients. Work carried out under this licence will also support the development of new methods and technologies to treat cancer patients. The development of new tumour models and/or improvement of existing models will allow us to test our experimental therapies in animals in a manner that more closely represents specific patient populations. Importantly, results may also be used to determine which drugs should not be progressed further, In addition to the outputs mentioned above, we expect to publish our results and to present our findings externally to scientific peers.

Who or what will benefit from these outputs, and how?

This programme of work is expected to enable us to progress new cancer medicines through progressive stages of development. Ultimately, if successful, these medicines will

be approved for use in the clinic to treat patients with cancer who otherwise are likely to die from their disease. Drugs developed using this licence therefore have the potential to benefit millions of cancer patients across the globe.

The development of new or improved cancer models will help us to target the right patient populations with our drugs which will increase the likelihood that those patients will benefit from the treatment.

The work carried out under this licence is also expected to contribute knowledge to the broader scientific community through the publication of our findings, presentation at external scientific meetings, and through collaboration with experts in the field.

How will you look to maximise the outputs of this work?

Our team has an excellent track record of publishing our advances in drug discovery and animal modelling. We aim to publish both successful and unsuccessful results in relevant journals and to share our data and learnings with collaborators to avoid others repeating work. In addition, we often present our research at international conferences for the benefit of the broader scientific community. These public discussions of our results have the potential to lead to new collaborations which provide additional opportunities to develop innovative new drugs and technologies.

Species and numbers of animals expected to be used

- Mice: We expect to use up to 51500 mice over the course of 5 years.
- Rats: We expect to use up to 800 rats over the course of 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice and rats are the most common animals used for the type of work that we are doing. They have immune systems that resemble those of humans, which means we can grow tumours in these animals to investigate the effect of our experimental therapies on the immune response to tumours. A large selection of different tumour cell types are available for use in mice and rats such as from lung, skin, or breast cancers. We can therefore evaluate our drugs in many different types of tumours.

In the vast majority of experiments we plan to use mice rather than rats. This is because there are more tumour types available for mice, and there is a larger body of published data for mice to help guide our experimental plans and interpret our results. Occasionally rats may be preferred such as when specific tumour types are available for rats but not mice, when an experimental drug is active in rats but not mice, or when we need to

compare our results with other data generated in rats. An example of this is safety data, where rats are often the preferred species.

We plan to use adult mice that are typically 6-12 weeks old at the start of the study, as the immune system is considered immature in mice younger than this age and therefore would not adequately represent the biology of the cancer patients we aim to treat. Occasionally we may need to use mice that are significantly older than this. This is especially relevant as cancer is generally a disease of middle to old age. For those occasions, we plan to conduct studies on animals up to 18 months of age as our previous experience has shown that this age is sufficient to see immune system changes as a result of ageing but before the mice begin to suffer serious effects of old age.

Rats used in this licence will also be adult stage, typically 6-12 weeks old, at the start of the study.

Typically, what will be done to an animal used in your project?

Animals are kept in high-quality facilities, free from pathogens (disease-causing organisms such as bacteria, viruses, and parasites) and with access to food, water, and environmental enrichment. In all the facilities used, the animal care staff are highly trained in rodent welfare and will ensure the animal suffering is minimised. Animals are housed in groups except in exceptional circumstances, for example when aggressive behaviour puts the welfare of the animal at risk or when cage-mates have been removed for experimental reasons.

Most of the animals will be used for studies that measure the impact of experimental drugs on tumour growth rates. The vast majority of tumours will result from injection of tumour cells under the skin which then grow into a tumour at the site of injection. However, in some instances we will introduce tumours into specific organs through specialised injection routes. For example, direct injection into breast tissue mimics breast cancer, while injection into the bloodstream leads to tumour growth in the lungs.

Producing tumour growth in other internal organs requires surgery, such as to implant tumours into the pancreas or sometimes into the breast tissue. Tumours that grow just under the skin are easy to observe and to measure their size using callipers. Tumours that grow internally are more difficult to observe therefore other methods are employed such as imaging techniques and/or clinical scoring systems to carefully monitor the well-being of each animal.

In some cases, it is not necessary to use animals bearing tumours. For example, tumours are often not needed to determine whether a drug is tolerated by the mice or to measure how much of a drug enters the bloodstream. In addition, we sometimes can use animals that do not carry tumours when we need to understand the impact of our drugs on specific aspects of the immune system.

On some studies, we will apply advanced imaging techniques (such as ultrasound or other imagers that can detect tumours or drugs labelled with special tracers) that allow us to track growth of tumour cells or to follow distribution of a drug throughout the body. To do so the animals will be anesthetised throughout the imaging session.

Blood samples or small samples of tumour tissue may also be collected during some studies to measure levels of drug or other indicators of drug effect over time. Blood samples are usually of a small volume and are taken from a vein in the tail or at the end of the study if larger volumes are needed.

Small pieces of tumour material are collected from live animals under anaesthesia with a fine-gauge needle.

Experimental therapies are most commonly injected into the peritoneal cavity (abdominal cavity), intravenously, or directly into the tumour. Occasionally drugs may be administered orally (typically by gavage, which involves the insertion of a tube through the mouth and down the throat) or rarely, subcutaneously (under the skin). At times these substances may only need to be administered once, but more often they are administered according to a schedule that requires multiple administrations. For example, drugs injected into the peritoneal cavity are typically given two to three times per week,

whereas drugs given orally would typically be administered once or twice a day, often for the duration of the study. In most cases our studies last approximately one month but on occasion when tumours grow slowly could last for 5-6 months. Therapies are most typically administered early in the day so that there is plenty of time to watch for any unwanted or harmful effects.

To understand the impact of our work on the animals, here are examples of a typical study and a worst- case scenario for our most commonly-used method in which a tumour is injected under the skin:

A typical study involves:

- implantation of a microchip under the skin for identification
- injection of tumour cells under the skin
- administration of experimental therapies by one or more of the following routes:
- into the peritoneal cavity (typically this will be done twice a week for three weeks)
- intravenous (typically this will be done once or twice a week for 3-4 weeks)
- orally by gavage (typically this is done once or twice a day for the duration of the study) collection of a blood sample

Worst-case scenario study:

- administration of experimental therapies by one or more of the following routes:
- into the peritoneal cavity (maximum 30 injections over the animal's lifetime)
- intravenous (maximum 14 injections over the animal's lifetime)
- orally by gavage (maximum 120 administrations over the animal's lifetime)
- injection of immune cells by one or more of the following routes:
- into the peritoneal cavity (contributes to lifetime limits for this route)
- intravenous (contributes to lifetime limits for this route)
- intra-tumoral (contributes to lifetime limits for this route)
- when animals are imaged (for reasons outlined above), this is done under anesthesia. The maximum number of times an animal may undergo anesthesia in order to be imaged is 5 times within a 7 day period, or 20 times in total over the animal's lifetime.

At the end of procedures, all animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

In our studies, the likeliest sources of adverse effects are from the size and condition of the tumour, from surgical procedures, and from the drug treatment. Animals will be classified as mild or moderate severity according to a scoring system that is based on the degree and duration of clinical observations such as body weight, activity level, posture, and body condition. This scoring system is currently under development, but will take into account the cumulative experience of the animal. For example, two clinical signs of short duration and low intensity might be classified as mild whereas the appearance of three clinical signs for longer periods of time and greater intensity would likely be classified as moderate.

We will humanely kill any animals that have developed large tumours to minimise unnecessary suffering through the use of well-defined tumour burden limits. Generally, studies with subcutaneous tumours will reach these limits within 30 days of implantation, but tumours that grow more slowly could take 2-3 months before reaching the same limit. The slowest tumour type that we currently work with takes approximately 5 months to reach the same size without any drug treatments that might inhibit the tumour growth.

Animals undergoing surgical procedures are assumed to experience pain due to the surgery and so will be classified as moderate severity. Pain relief or anaesthetics will be provided when surgery is performed.

Treatment of animals with cancer therapies may also lead to unwanted effects similar to those experienced by human patients. While humans may experience fatigue or fever soon after receiving the therapy, we observe similar responses in rodents such as reduced

mobility, hunched posture, and piloerection (bristling of fur). Animals that have been treated with vaccinia virus may also develop lesions that are usually found on the tail. Most of these effects will be of short duration and mild but some animals may experience moderate effects. We expect that approximately 30% of animals will be classified as moderate as a result of adverse reactions to drug substances, and the remaining 70% will be mild or lower severity.

At the end of procedures, all animals will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For both mice and rats, we expect the following proportion of severities under this PPL: Mild = 36%

Moderate = 64%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although we do many experiments using cells, molecular biology, and computer modelling, it is still necessary to use some animals for research so that we can more accurately assess the interaction of cancer cells with other cells and organs within the body. Isolated cells and organs do not reproduce the complex nature of in vivo (in a live animal) biology. The use of animals also allows us to understand cancer in the organ of origin or as it spreads throughout the body. An important aspect of our work is to understand how the immune system can be harnessed to attack tumours, and it is not possible to fully recreate these complex interactions outside of a living animal. In addition, regulatory agencies often require animal studies prior to approval of a drug for use in human clinical trials.

Which non-animal alternatives did you consider for use in this project?

Our organization regularly uses a range of in vitro (taking place in a test tube or laboratory dish, outside a living animal) methods. Such methods may include experiments containing a single cell type or multiple cell types grown together, which allows both direct interaction and indirect communication between cells. These types of studies are well-established, 2-

dimensional experiments that are useful to understand the specific ways that our experimental drugs affect cellular function.

More recently, we also have developed a more complex experimental system called 'tumour slice culture system' which involves the maintenance of thin slices of human tumour tissue in a dish. This method preserves the 3D structure of a patient tumour and is expected to be more representative of the biology of the whole tumour compared with the 2D experiments mentioned above. We are also investigating other 3D systems such as spheroids which allow cells in vitro to grow in all directions similarly to how they would grow in vivo, compared with traditional 2D methods in which cells are grown on a flat surface in a petri dish.

Why were they not suitable?

Cell-based methods are useful to gain an understanding of the way that our experimental drugs impact the function of different cell types outside the body, but do not adequately test whether the drugs remain stable after they enter the body, can reach the site of the tumour, or whether they are capable of inhibiting tumour growth in a live animal.

The 3D assay systems such as tumour slice cultures are a valuable addition to our experimental toolbox, however the slice cultures have a short lifespan. In addition, they can show modification of cellular function but do not demonstrate that this effect can shrink a tumour. The spheroid cultures are still being developed.

None of the alternatives investigated can demonstrate how our experimental therapies are broken down by the body, nor can they provide any information about how to schedule dose regimens. Unlike experiments using animals, they also do not permit identification of specific signals produced in the body which can be linked to tumour growth inhibition.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers were calculated based on our use over the last several years, and an expectation that we will increase the number of our studies by approximately 30% beginning this year. This is due to a planned expansion of our team and the number of projects under investigation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our organization includes a team of statisticians who have implemented a Good Statistical Practice program where animal experiments are formally reviewed before they start. This review is conducted by a researcher and a supporting statistician team to ensure the goal, experimental design, and data analysis align. This process ensures the design is robust and the minimum animals are used that are needed to answer the scientific question.

In addition, the following guidelines and online tools are also used to influence the design of our animal studies:

The PREPARE Guidelines, found at https://norecopa.no/prepare

The NC3Rs Experimental Design Assistant, found at https://www.nc3rs.org.uk/experimental- design-assistant-eda.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We ensure small scale pilot studies are carried out for new tumour types or experimental methods which enables us to design our studies using the minimum number of animals needed to achieve our scientific objectives. We also have implemented innovative study designs to reduce animal numbers where possible. We have also developed a new technique to take small samples of tumour tissue from live animals with a small needle, similar to a biopsy. This reduces the number of mice used for some studies because it avoids the need for separate groups of mice to both analyse the tissue and to monitor the growth of the tumour.

During our previous projects we have analysed the tumour tissues of the most frequent types of tumours that we grow in mice in order to define their cellular, molecular, and genetic characteristics. This characterisation has been important for reducing animal use since it ensures we can select the most appropriate tumour type for each experimental question, thereby reducing the overall number of experiments and allowing us to maximise the benefit gained from each mouse. A large amount of data has been generated from our characterisation work and is archived for use by all our scientists to guide the design of future animal studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The most common type of tumour that we use involves injection of tumour cells under the skin resulting in tumour growth at the site of injection. This is the simplest method available to grow tumours in rodents and carries the least welfare risks since the tumours are not located in vital organs. They are also the easiest to monitor since they can be easily observed and measured under the skin. This method is preferred except in specific cases when we need to understand more complex questions. For example, we may need to understand the spread of cancer from one site to another, the influence that specific cell types and organs have on tumour growth, and the responses of tumours to our therapies in these varied settings. In these cases, we will use more complex experimental methods in which tumours often develop inside the body cavity. These more complex studies also may involve surgical implantation of tumours. Where possible, non-surgical methods will be used for implantation and imaging methods used to monitor tumour burden.

Why can't you use animals that are less sentient?

Rodents are the lowest species of mammal (meaning they are the least sentient, or least aware of feelings and sensations) that allow us to adequately study the complexity of human cancer and immune system biology. Because many of our experimental therapies are designed to impact the immune system, it is essential that we use adult animals with mature immune systems in our research. Our studies also monitor the growth of a tumour over a period of weeks to months therefore it is essential that the animals are conscious as the use of anaesthesia (an agent that induces a state of unconsciousness) would not be possible for such an extended period of time. In addition, the behaviour of conscious animals also often alerts us to adverse reactions to our therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are committed to refining our procedures to minimise harm to the animals and have a track record of doing so. We ensure small-scale pilot or tolerability studies are carried out for new tumour types, experimental methods, or therapies. We carefully monitor tumour burden including the use of whole- body imaging techniques when possible. We also use tumour-free mice in some cases when tumours are not essential. We have implemented innovative study designs to reduce animal numbers and enhanced health checks to minimize suffering. General welfare of the animals is assessed by checking body weight and watching for the development of clinical signs such as activity levels, appearance of the coat, posture, and body condition. When dosing animals with test substances, we dose as early in the day as is practical in order to maximise the length of time that animals are monitored during the day. When unexpected events occur, these will be thoroughly investigated to find out what happened so action can be taken to prevent a reoccurrence. When two or more drugs are scheduled to be injected at the same time, we will also combine them into the same syringe where possible in order to minimize the number of injections administered to the animals. In addition, a non-surgical method of tumour implantation in the mammary fat pad has been developed and is used in preference to surgical methods wherever possible. We have also introduced the use of an improved

method for judging the size of a tumour growing inside the body cavity in our pancreatic tumour studies. This involves the use of a standard set of clay balls of different sizes. The feel of the tumour under the abdomen can be compared against the clay balls to determine the approximate size of the tumour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our practices are principally guided by the Workman et al guidelines published in 2010 (Workman, P et al. Guidelines for the welfare and use of animals in cancer research. 2010. British Journal of Cancer.

102 p1555). In addition, we follow the ARRIVE guidelines developed by NC3Rs for publication of our work in peer-reviewed journals (ARRIVE Guidelines version 2.0 released in July, 2020 can be found at https://arriveguidelines.org/) and our practices incorporate many of the guiding principles of the PREPARE guidelines (Smith et al., PREPARE: guidelines for planning animal research and testing.

2017. Laboratory Animals.) LASA (Laboratory Animal Science Association) also has a range of published guidance documents with principles that can be applied to our animal studies which are found at https://www.lasa.co.uk/current_publications/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our team is actively involved in promoting the 3Rs and participates in global 3Rs activities including an annual poster competition. This award was given to individuals working on the development of alternative models under a previous project license in 2009, and also a team working on an ex vivo (taken directly from a living organism) tissue slice culture system in 2017. Our team also is aware of advances in 3Rs through the NC3Rs and establishment websites and via participation in conferences and events sponsored by organizations such as LASA (Laboratory Animal Science Association, IAT (Institute of Animal Technology) or NC3Rs.

10. Non-invasive mucosal rodent immunisation

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- (iii) Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

vaccine, oral, nasal, inhalation, non-injectable

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to produce safe and effective non-injectable vaccines.

We have a track record in this, and our vaccines will be developed using platform mucosal delivery systems, initiated through our previous research. These delivery systems can be

applied to a wide- range of conditions, designed and optimised using computer modelling to prevent infectious and non- infectious disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vaccines have been the main focus of the World's attention since the COVID-19 pandemic; highlighting their importance in fighting infections. However, while many companies and researchers initially focused on injectable vaccines, products that can be administered by non-invasive routes (eg via the mouth, nose, lungs) have remained a desire by the pharmaceutical industry. These so-called mucosal vaccines can improve effectiveness by protecting the routes of entry of infections (eg skin, mouth, nose, lungs - also known as mucosal surfaces) as well as the whole body (eg via the blood stream), can provide better distribution options to vaccine centres as they don't need cold storage, are safer to administer and are more user-friendly (without the use of needles).

What outputs do you think you will see at the end of this project?

1. New basic information about the mucosal immune system (poorly understood as most vaccines are given via injection and better understood).
2. Understanding and design of optimised vaccines using computer modelling to reduce use of animals in the future.
3. Peer-reviewed publications, conference presentations, postgraduate student theses.
4. Proof-of-concept data to enable grant applications and commercialisation to develop safe and effective products.

Who or what will benefit from these outputs, and how?

The overall purpose of this research is to develop safe and efficacious mucosal vaccines to protect against infectious disease and to prevent non-infectious conditions.

1. Short-term - Animal welfare for animals in vaccine design will be improved as the administration methods are non-invasive
2. Short-term - Scientists will benefit from basic information about the mucosal immune system.
3. Short-term - On the current mucosal licence, we are developing the following mucosal vaccines: anti- COVID-19 nasal vaccine, anti-Myeloid Myeloma (blood cancer) oral vaccine, antibiotic-free oral products for poultry, anti-fertility vaccines for squirrel control, anti-fertility vaccines for rodent control.

4. Long-term - Humans and animals will benefit from any vaccines developed as they will be less invasive than the current injectables and could be used in situations where the current injectables cannot be eg very young children and the elderly.
5. Long-term - Roll out of vaccines will be much easier as mucosal vaccines can be designed without the need for cold storage.
6. Long-term - Over the next 5 years, we anticipate we will attract commercial interest in our platform delivery systems to continue to make an impact to the field of mucosal vaccines. Most our work has been on oral and nasal routes, with less work carried out on topical, pulmonary and vaginal - depending on the diseases/conditions being studied.

How will you look to maximise the outputs of this work?

Applications for grant funding is being carried out with companies and organisations that will be able to take these ideas forward. We currently have pilot studies being carried out on a previous licence - these will be progressed in collaboration. Dissemination of new knowledge will occur once intellectual property is protected, through publication, conferences and interested networks. When we publish, we will use the ARRIVE guidelines so the work is reproducible.

Species and numbers of animals expected to be used

- Mice: 250
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For the last two decades my research has focused on mucosal vaccines. Most of this work was initially carried out on rodents and then progressed to larger species such as ferrets, dogs and sheep through the funding bodies.

After in-vitro screening adult rodents have always been used for initial screening, and has been essential for anti-fertility applications. Mice have been used to screen antibody levels, but rats have been used for hormone and fecundity testing (and found to be more reliable).

Typically, what will be done to an animal used in your project?

The purpose of developing mucosal vaccines is to reduce the stress to humans and animals. Therefore, the main criterion for these products is that they minimise animal suffering in experimental models and that they are “mild” in severity. In addition, the pharmaceutical industry requires as few administrations as possible, therefore our

research will also take this into account in the experimental design. Some of the administration routes require anaesthetic and this is to minimise mis-administration rather than because they cause pain.

Typically 3-4 administrations (each not taking more than a few minutes) will be given over a 2 month period. Pre-administration animals may have food withdrawn for short periods to prevent interaction of vaccine with food if administered orally. Following administration, tail bleeds will be taken a week later for antibody and hormonal measurements. This is a quick process not taking longer than 5 minutes. In some cases drugs may be administered in drinking water to enable control of stomach acid (to prevent interaction of vaccine) or to cause salivation to enable more mucous to be produced to analyse them for protective antibodies. In some types of fertility experiments breeding will be set-up or vaginal smears will be taken (this is a quick process taking no more than a few minutes).

Some animals will experience traditional routes of administration such as subcutaneous and intramuscular and are required as a comparison to the mucosal route results.

What are the expected impacts and/or adverse effects for the animals during your project?

Anaphylaxis caused by antigen administration is possible but rare. To avoid this formulations will be tested in-vitro for toxicity before administration. No adverse effects expected from pre-treatment (eg ranitidine), as it is routinely used in this type of study and from our previous experience.

Injections (for control comparison with mucosal routes): For injections under the skin - minor swelling and redness at the site of injection. Good handling to minimise discomfort and observation after dosing with humane killing of any animal showing major skin ulceration (e.g., non-healing or deep or recurrent ulcers or secondarily-infected ulcers). For injections into the muscle (which we will avoid as far as possible), lameness may occur but this should be mild and short-lived.

Mouth administration: Mild irritation in lining of mouth may occur. If this happens, soft food will be provided.

Oral dosing into the stomach: Minor discomfort at time of dosing, rarely the substance may enter the airway or the oesophagus is damaged (less than 1 in 1000 administrations). Good handling to minimise discomfort and observation after dosing with humane killing of any animal showing signs of mis-dosing or damage. We will also attempt to reduce discomfort by oral dosing through incorporating vaccine into drinking water/food. Any animal showing signs of weight loss will be brought to the attention of animal care staff and, if necessary, the NVS. Non-reversible weight loss going beyond 10% of the animal's initial body weight will constitute an endpoint.

Nasal dosing: Short lived nasal irritation. Increased respiratory rate, which is short-lived and observed in less than 1 in 1000 animals. Good handling to minimise discomfort and

observation after dosing. Any animal showing marked respiratory distress will be immediately culled.

Topical: Mild redness. Possibly mild swelling and discomfort lasting only a few hours. Swelling observed in less than 1 in a 1000. If it persists or the animal shows significant discomfort, NVS advice will be sought.

Vaginal: Short lived mucosal irritation. Good handling and technique to minimise discomfort.

Inhalation: using a nebuliser. Mice will be treated without using an anaesthetic using a Volumetric Spacer or a suitable commercial holder. Rats will be treated by direct nebulisation with/without an anaesthetic or by using a Volumetric Spacer or a suitable commercial holder. In order to carry out pharmacokinetic studies we propose to use a microsprayer system.

Administration of formulation – To reduce discomfort as much as possible, volumes and frequency of drug will not exceed the recommended maximum volumes for the different routes following local good practice guidelines.

Pilocarpine administration results in an increased salivary production, but this effect is transient and the animals tolerate it well.

Anaesthesia – The risk of death from anaesthesia will be minimised by the use of experienced personnel and proven techniques. Anaesthetic mortality is not expected to exceed 1%.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All procedures are expected to be mild for both mice and rats.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The ultimate end of this work is to develop new mucosal vaccines primarily for use in humans but also animals. In order to proceed to human or large animal trials it is

necessary to pre-screen these new vaccines in rodents in order to ensure that they do induce suitable antibody levels.

Which non-animal alternatives did you consider for use in this project?

We have performed database analysis for possible alternatives but there's currently no non-animal substitute for this type of work. We have introduced computer modelling and also use in-silico testing to design our vaccines; this helps replace and reduce some use of animals by only proceeding with in-vivo work when we have identified the best candidate vaccines/compounds.

Why were they not suitable?

Computer modelling and in-silico tests can't currently replace or recreate the complexity of the immune system and, in particular, the mucosal immune system for which less knowledge is available in terms of vaccination.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The establishment statistician has been consulted and based on previous licence use we will use 5 animals per test group.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I have always kept animal use to a minimum by careful experimental design in close association with the establishment's statistician and the numbers used give sufficient data to enable vaccine products designed to progress to the next stage of development.

Protocols have also been improved by using in silico modelling to help design our formulations so that fewer experimental groups need to be used

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cell work and computer modelling will be carried out first.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

- Which animal models and methods will you use during this project?
- Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The vaccines being developed are designed to cause the least possible pain, suffering, distress to the end-user, be they people or animals. Therefore, the methods of administration used in this project are not expected to cause pain or suffering other than mild, transient discomfort. These models (rodents) are used as an initial screen and are necessary in order to progress to the next stage of development.

Why can’t you use animals that are less sentient?

Mature animals are needed to obtain the appropriate responses in terms of antibody, hormone, fertility.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are trying to design better vaccines that will reduce the number of administrations and this will be a key goal that will minimise welfare costs and inform future work.

For oral administration, instead of gavage, we have also tried voluntary oral dosing in the past using sweetened chow and will use this whenever possible. Other methods we will consider are outlined in these publications:

https://doi.org/10.1016/j.bbi.2020.04.015
https://www.sciencedirect.com/science/article/pii/S266616672100037X
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4587624/

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs guidelines on administration of substances, our own local dosing guidelines, the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am very active in the field of mucosal vaccines and am kept informed by reading and publishing work in this area.

In addition, I use the NC3Rs webpages and Named Information Officer at the establishment.

11. Normal and leukaemic blood cell development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

haematopoiesis, blood cancer, ontogeny, therapy

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how human blood cell development (haematopoiesis), changes through the human lifetime, and how this affects the biology of blood cancers (leukaemia) at different ages.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Blood cell production is a precisely controlled process that starts before birth and continues through the human lifetime. Although adult blood cell production has been studied; not much is known about how blood cells develop in fetal life. These studies will provide us with detailed cellular and molecular mechanisms of how normal blood cells are produced, renew and differentiate throughout ontogeny.

There is growing evidence that many childhood leukaemias start to develop in fetal life. Some rare leukaemias such as those that develop very early (<12months of age), are called infant leukaemia and these children tend to do poorly even with intensive treatment. Newer treatment strategies are urgently needed for treatment resistant infant/childhood leukaemias. To enable this, a more detailed understanding of how and when these leukaemias develop, and whether the biology of treatment resistant disease depends on the developmental stage the leukaemia originates in is crucial. However, the specific properties of fetal cells that make them prone to undergo leukaemic transformation, and the exact cell of origin of childhood leukaemias is not known. This project will i) help identify the prenatal target cell that undergoes leukaemic transformation in infant and childhood acute leukaemia ii) create faithful mouse models of leukaemia using human prenatal and postnatal haematopoietic cells. This will aid in better understanding of the pathogenesis of refractory leukaemia at different ages, and also develop novel therapies for the same.

What outputs do you think you will see at the end of this project?

New information generated at the end of the project:

Detailed characterisation of how blood cells develop throughout the human lifetime.

Creation of novel leukaemia models by transforming human haematopoietic stem and progenitor cells.

Testing novel therapies in the models developed to target key vulnerabilities in treatment resistant leukaemias

All data generated will be shared via publications. They will also be deposited in publicly available repositories after publication, and will form a valuable resource for the scientific community.

Who or what will benefit from these outputs, and how?

The short-term beneficiaries from this study would be scientists studying human haematopoiesis and/or the biology and pathogenesis of childhood leukaemia. Data generated will help in the understanding of the pathogenesis of these diseases. Results will be disseminated at national and international Haematology meetings and via scientific publications. However the main beneficiaries of this project will hopefully be infants and children with abnormal haematopoiesis and/or leukaemia because of a) better understanding of the origins of the disease and b) testing of novel treatments that target

specific molecular pathways identified. It is anticipated that it will take at least 5 years to develop therapies that can reach clinical trials. We will focus on a poor prognosis subtype of childhood leukaemia that occurs in infants <12 months initially, with a view to studying other treatment resistant subtypes in the future.

How will you look to maximise the outputs of this work?

This project forms part of many collaborative studies that are underway with national and international collaborators studying normal and abnormal haematopoiesis. We work in close collaboration with clinicians in hospital settings, clinician scientists, scientists and computational biologists in UK Universities in order to maximise the academic impact of this work. Any new methodology that is established will be shared with the research community. The sharing of knowledge and implementation of novel treatment strategies in national and collaborative international trials will help deliver the benefits of research at a global scale.

Publication of results in highly visible journals and adhering to the funder's policies regarding open access is one way we will clearly communicate our results to a wide audience including other researchers and clinicians. This will include negative results. Along with publication, datasets will be deposited into public repositories so that the raw data will be available to other scientists for re- analysis. We will also make a specific effort to communicate our results to clinician-scientists by presenting our results at conferences both at a national and international level. Publications and presentations will be accompanied by an appropriate summary in the public science arena. Prior to significant publications we will liaise with funding bodies and other media sources for preparation of press releases to the media, so that the dissemination of the research extends to as wide an audience as possible.

Species and numbers of animals expected to be used

- Mice: 7000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Blood formation and leukaemia development are precisely controlled processes that require the living environment, such as bone marrow. Immature blood cells called haematopoietic stem cells (HSCs) are known to interact with other bone marrow cells, and when exposed to culture (non-living conditions), they change their properties. Therefore, these processes have to be investigated using animals. The mouse is the most widely used system to study the formation of normal blood and blood cancers.

Mouse models have demonstrated to be highly relevant and essential for development of an understanding and clinical application of the blood forming system in man, including the application of bone marrow transplantation and understanding of leukaemia since mouse and human stem cells share similar properties. Other advantages of the mouse model (apart from it being mammalian) include the availability of laboratory reagents to study blood functions. Furthermore, leukaemia models in mice can be used to understand the origins of the disease and to develop/ test potential targeted therapies.

Typically, what will be done to an animal used in your project?

Mice may undergo non-lethal dose of irradiation in preparation for transplantation of genetically altered or leukemic cells. They will also undergo blood sampling at regular intervals and on some occasions bone marrow samples may be taken from the femur. Mice will develop leukaemia and in some cases will receive treatments that may modify the disease. Typically this will be by injection but maybe administered in the food or drinking water.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts include transient discomfort or pain when substances are administered, when cells are injected, and blood or bone marrow samples taken. Irradiation of normal, immune- compromised or genetically modified mice can be associated with temporary (approximately 2 weeks) decrease in blood cell production and immune deficiency. The resulting transient low platelets and white blood cells starting from 5-7 days, can cause acute bleedings, anaemia and infections, just as seen in patients undergoing bone marrow transplantation. Some animals might also suffer from transient diarrhoea. Animals transplanted with malignant cells may develop leukaemia or high-grade lymphomas. Leukaemia will be recognised by elevated white blood cell counts (greater than 20% blast cells). Mice may develop anaemia, bleeding, or an enlarged abdomen (defined as >10% increase in body girth).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mouse:
- Mild: 25%
- Moderate: 75%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Blood formation and leukaemia development are precisely controlled processes that require the living environment, such as bone marrow. Immature blood stem cells are known to interact with other bone marrow cells, and when exposed to culture (non-living conditions), they change their properties.

Therefore, these processes have to be investigated using animals. The mouse is the most widely used system to study the formation of normal blood and blood cancers. Mouse models have demonstrated to be highly relevant and essential for development of an understanding and clinical application of the blood forming system in man, including the application of bone marrow transplantation and understanding of leukaemia since mouse and human stem cells share similar properties. Other advantages of the mouse model (apart from it being mammalian) include the availability of laboratory reagents to study blood functions. Furthermore, leukaemia models in mice can be used to understand the origins of the disease and to develop/ test potential targeted therapies. Some aggressive childhood leukaemia subtypes have so far been impossible to recapitulate. Our group has used human prenatal blood cells to model the disease for the first time and this will provide valuable information about how these leukaemias develop and pathways that drive this disease. This model and other new models generated will also be used to test novel targeted treatments in this project.

Which non-animal alternatives did you consider for use in this project?

We will perform a large number of analyses in non living conditions, such as checking how normal and abnormal blood cells grow in the lab and how rapidly; as well as detailed characterisation of their properties, such as genes that are active; and only use animals where these experiments are not possible, or will not allow us to address key questions. The protocols performed have been limited to those for which there are no alternative methods that will allow us to achieve our objectives.

Why were they not suitable?

Attempts to model or maintain haematopoiesis in cell culture have demonstrated that the properties of early haematopoietic cells change so that they prematurely lose their key

functions of self-renewal, and multilineage differentiation. Therefore, some of the objectives listed can only be achieved with the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of mice include those required for primary, secondary and tertiary transplantation of human haematopoietic stem and progenitor cells and leukaemic cells.

The experiments planned for this license are similar in nature to experiments currently performed in my laboratory, and animal numbers have been calculated based on experiments previously performed and also on the different types of treatments we will use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Investigators will carefully plan experiments to ensure:

Each experiment uses the minimum number of animals required to obtain a statistically meaningful result as determined with power calculations using the NC3R's Experimental Design Assistant.

Breeding strategies are designed to minimise the number of non-experimental mice generated

Mice are sourced from central colonies, and we will collaborate with other groups to ensure any surplus animals from their breeding colonies can be used, to avoid redundant breeding within this project.

For pharmacological studies we will use pilot studies to determine effect sizes, and experimental block design to avoid overpowering the analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Good colony management practices will be followed to ensure that only animals needed for experiments will be produced. Use of a database, which yields reports on colony performance data will be used to assist in colony management.

Maximising yields of haematopoietic cell populations: We have put extensive effort into maximising the number of the cells we can obtain from each animal by rigorously testing the optimal antibody staining concentrations, centrifugation speeds and enrichment methods to ensure that we minimise the number of animals used to obtain the cells. We will plan experiments within the lab, and where relevant across labs, to share tissues and cells from mice.

Molecular analysis on lower numbers of cells: We have developed technical and next-generation sequencing approaches leading to a major reduction in the numbers of cells required for each analysis, thus consequently dramatically reducing the number of mice required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice that are engrafted with human haematopoietic cells: Xenografting is currently the most refined model for studying normal human and malignant cells in a physiological setting. It does not cause any significant lasting harm, except those associated with disease progression in case of engraftment of malignant cells. It is associated with transient discomfort due to irradiation where used; however, this is required for ablation of the endogenous haematopoietic cells which in many cases is a prerequisite for the transplanted human cells to engraft.

Mice that develop haematological malignancies: The mouse currently represents the most accurate model for the experimental study of human haematological malignancies, and the resulting phenotypes are good representatives of the human conditions. For disease phenotypes it is necessary to allow the disease processes to proceed to a clinically relevant stage, which may cause the animal some pain and discomfort for a limited time. However, this is required to obtain information relevant to human health, and will be minimised by frequent monitoring and ending the experiment as soon as an adverse clinical phenotype develops.

Mice that are treated with pharmacological agents: To develop pharmacological strategies to treat haematological malignancies the use of drugs is required. Some drugs that are used to treat haematological malignancies are cytotoxic, and may cause transient pain and discomfort, but no lasting harm. However, their use is necessary to determine if treatments

are able to improve on the current standard of care, and the least toxic alternative will be used.

Why can't you use animals that are less sentient?

The mouse is the least sentient model with a haematopoietic system that sufficiently supports human haematopoietic cells for the study of normal blood cell development and malignancy to be relevant to human health. Blood formation and leukaemia development are precisely controlled processes that require the living environment, such as bone marrow. Immature blood cells called haematopoietic stem cells (HSCs) are known to interact with other bone marrow cells, and when exposed to culture (non- living conditions), they change their properties. Therefore, these processes have to be investigated using animals. The mouse is the most widely used system to study the formation of normal blood and blood cancers. Mouse models have demonstrated to be highly relevant and essential for development of an understanding and clinical application of the blood forming system in man, not the least application of bone marrow transplantation and understanding of leukaemia since mouse and human stem cells share similar properties. Other advantages of the mouse model (apart from it being mammalian) include the availability of laboratory reagents to study blood functions. Furthermore, leukaemia models in mice can be used to understand the origins of the disease and to develop/ test potential targeted therapies. Xenograft assays require transplantation of human cells into live animals. We will use adult mice for most xenograft assays. We will use neonatal mice only if required to develop particular types of early life leukaemia models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When undertaking work with animals to achieve our aims we have carefully chosen the least severe procedures to minimise the pain and adverse effects for the animals. Specific examples of refined procedures in the laboratory include:

Conditioning of haematopoietic cell transplant recipients with split radiation dosage: In order to detect the activity of the transplanted cells, the host animal's own haematopoietic system must first be depleted by irradiation, in the same way as is done with humans receiving bone marrow transplantation as a therapeutic modality. In order to minimise the morbidity and mortality associated with irradiation, a split of two half doses of irradiation, rather than a single full dose which, in association with temperature and noise monitored housing, provision of moist food and extra bedding, and rigorous monitoring has resulted in very low levels of morbidity and mortality. Recipient mice will be given antibiotics to prevent infections.

Administration routes: Cells, drugs and biologically active agents will be administrated by the least invasive route in the first instance when multiple routes are available for example orally in water or feed rather than by intraperitoneal route. Procedures that are likely to

cause more than transient discomfort will be performed under anaesthesia with adequate cover with pre and post operative painkillers.

Aseptic techniques will be used for administration of substances with special consideration to maintaining body temperature and hydration. If multiple subcutaneous injections are required, a subcutaneous device will be installed that can deliver the multiple doses, instead of repeated injections. For experiments that involve treatment of haematological malignancies to model the clinical standard-of-care we will define the least harmful treatment type that achieves clinically relevant results.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Surgery will be performed following LASA aseptic surgery guidelines.

We will use the NC3Rs Experimental Design Assistant (EDA) online tool or similar software to design experiments.

We will conduct and report our experiments for publication according to ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attendance at local animal welfare meetings is required. The NC3R's newsletter is reviewed and relevant points discussed with all members of the team. Attendance at local 3R's events is also encouraged.

12. Generation of new anti-tumour strategies for epithelial cancers

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Virus, Cells, Immune system

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to support the development of potential new cancer treatments and to understand the effects and mechanism of action of our anti-tumour drug candidates when administered alone or in combination with other therapies, some of them currently used in clinics. Our drug candidates aim to help the immune system to fight against the cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how our drug candidates work after systemic administration would help in selecting the best candidates to progress with to better address specific strategies (for example choosing the appropriate combination therapy or cancer indication) to treat cancer patients.

What outputs do you think you will see at the end of this project?

EnAd Our drug is an oncolytic (cancer-killing) virus currently being evaluated in clinical trials to treat a range of late-stage epithelial (such as breast, colorectal, lung) cancers. In these trials, EnAd it has demonstrated a good safety profile as well as a successful delivery to disseminated tumours, and there are indications that this may be associated with a favourable anti-tumour immune response. Under the previous licence, variants of the virus have been studied: those variants were designed to offer improved anti-tumour efficacy through enabling the virus to produce therapeutic agents within the tumour to assist the immune system in identifying the tumour as a foreign body and fighting against it. Currently three of these mentioned engineered versions of EnAd (T-SIGn viruses)this virus have entered clinical trials evaluating their efficacy at treating a range of late- stage epithelial cancers, with encouraging early data emerging. This project aims to generate more mechanistic data to better understand how our drugs work systemically, to be able to identify the best combinations with currently available therapies to treat different cancer's indications for which few or no other treatment options are available. In addition to this, under this licence new variants of EnAd our virus will be studied and the most notable potential benefit would be the successful translation of those engineered versions into the clinic and their subsequent development towards approval for the treatment of patients.

Who or what will benefit from these outputs, and how?

Under this licence, the quickest potential benefit would be the identification of good combinations of EnAd variants of our virus and other therapies currently used or being evaluated clinically, which will, in the longer term, lead to improved treatment options for patients with a range of late-stage epithelial cancers.

In addition to this, we aim to successfully develop new mechanistically different T-SIGn viruses versions of the virus for translation into the clinic and their subsequent approval for the treatment of patients with cancers for which few or no other treatment options are available.

How will you look to maximise the outputs of this work?

Results obtained from the work carried out under this licence will be broadly discussed both internally and with collaborators, to generate new available knowledge and to be able to deliver successful approaches into the clinic. Where possible, we will also seek to publish new data and associated methodologies and experience through presentations at conferences, submission to peer-reviewed journals etc.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Full evaluation of our drugs needs to be addressed in a living system at the age of developed mature immune system and mice are the species with the lowest neurophysiological sensitivity in which our work can be carried out.

Typically, what will be done to an animal used in your project?

Typically, each mouse would experience the following procedures - or less than the ones listed below depending on the experiment:

Chemoablation followed by injection of human stem or immune cells (less than 2000 mice will experience this under this licence)

Implantation of tumour cells (and then 3-4 weeks for cells to grow)

Induction and maintenance of anaesthesiaanaesthesia for tumour volume measurement (maximum 3 days per week)

Administration of test substances

Blood sampling (up to 10% blood volume may be collected on a single occasion, but no more than 15% in any 28-day period)

Approved humane method of killing or cardiac bleed under terminal anaesthesiaanaesthesia

What are the expected impacts and/or adverse effects for the animals during your project?

The most common expected adverse effects will be tumour ulceration, whereby small wounds can develop on the surface of the tumour, and weight loss following chemoablation of mice where mice are reconstituted with human immune cells. Weight loss will be managed using replacement diet, such as fluid therapy orally administered via a gel diet. Observations made during post-mortem analysis of killed mice will be used to refine models and tighten humane endpoints to ensure suffering is always minimised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All the work carried out under this licence will be of moderate severity or below.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Full evaluation of our drugs - such as the systemic delivery, as well as the recruitment and activities of cells of the immune system to destroy the cancer in situ - rely on complex interactions and need to be addressed in a living system.

Which non-animal alternatives did you consider for use in this project?

The transition of experiments into animal models follows on from extensive in vitro and ex vivo (taking place in a test tube, culture dish, or elsewhere outside a living organism) work to ensure each candidate variant of the drug is fully functional, as well as 'safe'. This includes a huge amount of work performed with cell lines, and primary cells from either healthy donors or cancer patients - with informed consent.

This ensures that no animal work is carried out where the desired information could be obtained via alternative methods and that in vivo (taking place in a living organism) studies are designed around a deeper understanding of our drugs being assessed. This limits the number of mice required under this licence, but it cannot replace them entirely.

Why were they not suitable?

There are some mechanisms which can't be assessed with in vitro or ex vivo experiments: for instance, the recruitment of immune cells to the tumour microenvironment, or the biodistribution of the virus particles/transgenes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is a rough estimation based on the objectives detailed under this licence and the studies planned for the foreseeable future, although it does of course take into account the projection of our research over the next 5 years.

Extensive planning and regular in vivo meetings within our research team and external collaborators will ensure that we get the most out of each study, minimising the number of animals used but without compromising the experimental outcome (minimising need for repeating experiments and ensuring the appropriate number of mice is used to get significant results).

Calculations has been made considering that we estimate to run studies with 60 mice each - this is a number which allows us to cover all the experimental conditions. On average across the 5 year of this licence, we consider that as a team we will run 8 of these kind of studies per year, plus possibly a couple pilot studies (with 20-30 mice each).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Biostatistics are carried out prior to embarking on studies, utilising data acquired in pilot (or similar previous performed) studies to ensure that group sizes and randomisation are appropriate to minimise the need for repeating experiments and to ensure that excessive (or too low and therefore risk for inconclusive results) numbers of mice are not used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Pilot studies will be performed to ensure the correct setup for new experiments.
- Once we implant mice for a study, we aim to obtain the maximum amount of information from it: therefore, when the humane end point is reached and the mouse is killed by schedule 1, several readouts are planned.
- By investigating new approaches utilising ‘in-house humanised mice’ or in vitro cultured immune cell transfer we hope to reduce mouse to mouse variability and therefore reduce the numbers of mice required to confirm an anti-tumour effect.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All worked carried out under this project licence will utilise mice.

A great deal of care is taken to ensure that our studies are as refined as possible, to minimise suffering. The process of refinement runs in parallel to all of our in vivo studies to ensure that even 'tried and tested' models are subject to scrutiny, and to enable the application of more appropriate humane end points wherever possible.

Why can't you use animals that are less sentient?

Full evaluation of our drugs - such as the systemic delivery, as well as the recruitment and activities of cells of the immune system to destroy the cancer in situ - rely on complex interactions and need to be addressed in a living system with fully mature immune system to mimic what would happen in the human body.

Mice are the species with the lowest neurophysiological sensitivity in which our work can be carried out, and many mouse models are readily available and extensively studied which can be used to bridge the gap between mouse and human immunology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During the experimental design carried out under this licence, we will deeply consider both the welfare refinement (to improve the animal experience) and the scientific refinement (to improve the data collection). Every detail of those aspects matters for the refinement of the entire animal experience.

General procedural refinements applied to studies carried out under this project licence include:

- Treatment will always be delivered at the smallest effective delivery volume
- Warm boxes will be used to maintain body temperature where extended anaesthesia is required and to dilate blood vessels for injection to minimise bruising
- After administration of substances, animals will be carefully monitored during the whole study
- Post-mortem analysis of mice at the end of studies enables us to better understand our models and to apply tighter humane endpoints wherever possible
- Human blood utilised in studies bridging the gap between human and mouse is obtained from clinical sites that allow for the same donors to be ordered repeatedly. Another approach that we are developing
- in-house is the generation and expansion of immune cells (from human blood) already 'committed to react against a specific target'. This avoids toxicities associated with the immune cells from human donors adversely reacting with mouse tissues.

- Animal welfare meetings held with other local licence holders enable the sharing of information so we can all ensure our studies are designed around a broad knowledge of current best practice to ensure the minimum welfare cost for the animals (considering for example the amount and frequency of cells injected into the mice according to the best outcome for desired results)

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA guidelines NC3R's

RSPCA UFAW

Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and surrounding animal legislation

Guiding principles on good practice for Animal Welfare and Ethical Review Bodies PREPARE guidelines

ARRIVE guidelines UKCCR guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Useful webpages kept under consideration:
https://www.understandinganimalresearch.org.uk https://www.animal-ethics.org
https://www.nc3rs.org.uk

Also, I we will be constantly in contact with NACWO and vets to ensure the best practice is always performed.

13. Blood clot properties in thrombosis, thromboembolism and atherosclerosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Thrombosis, Blood coagulation, Thromboembolism, Fibrin, Clot structure and function

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Recent studies in patients with heart disease and in patients with clots in their leg, brain or lungs identify the structure of the blood clot, and its stability, as a potential cause of the disease.

Currently it is not known which clot architecture is optimal in terms of providing normal clotting at a site of injury, or after surgery, while at the same time reducing any risk of developing a blood clot in the heart, brain, lungs or leg. The aim of this project is to analyse which clot structure(s) support optimal blood vessel repair and reduce the risk of disease-causing and ultimately deadly clots.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gaining knowledge about which clot structure is optimal blood vessel repair, but reduces the risk of clots in the brain, heart, leg or lungs (thrombosis), will enable improvements in treatment of conditions where clotting plays a role. A critical areas of patient benefit is likely to arise from finding out which clot structures reduce the risk of thrombosis to enable the development of new drugs that target clot structure, making them easier to breakdown or remove using catheter mediated clot removal devices.

What outputs do you think you will see at the end of this project?

Studies focused on clot architecture will lead to an improved understanding of its role in determining thrombosis rates, thrombus size and resistance to thrombolysis by clot busting drugs.

Models on clot embolization will lead to an improved understanding of the role of clot structure in clot instability and embolization, both in terms of pulmonary embolism and ischaemic stroke.

Studies focussed on clot structure and crosslinking will deliver on understanding the roles of crosslinking in inflammation driven thrombosis, and understanding the roles of crosslinking in atherosclerosis and atherothrombosis.

Together, these outcomes will elucidate processes that underpin pathological clot formation during thrombosis, thromboembolism and atherosclerosis.

Who or what will benefit from these outputs, and how?

New models of thromboembolism will benefit researchers interested in such models around the world. New models of thromboembolism may also be of future interest for the pharmaceutical industry, for the testing of anticoagulant or thrombolytic drugs. Clinicians and patients will ultimately benefit in the form of improved treatment and care based on clot structure and stability.

Short-term, medium-term and long-term benefits:

Short-term: Increasing and disseminating new scientific knowledge in the field of thrombosis and bleeding disorders by peer reviewed publications in scientific journals, presentation of data at national and international scientific meetings, press releases on research outcomes where appropriate.

Medium-term: Assistance of other interested researchers in new models and techniques. Sharing of models, strains and reagents with other groups, nationally and internationally.

Long-term: Improvements in treatment and care of patients with thrombosis and thromboembolism. Note that these long-term benefits are beyond the timeframe of this project licence application.

How will you look to maximise the outputs of this work?

We will disseminate outputs to the academic audience through national and international scientific meetings and workshops. We will further disseminate via publication in peer-reviewed journals. We will also communicate outputs to the lay audience as appropriate through press releases.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Choice of species. The mouse is a useful model for preclinical modelling of diseases of thrombosis, thromboembolism and atherosclerosis. This is because of the large number of available genetically- modified models, basic similarity between mechanisms of haemostasis, thrombosis and vascular wall pathologies in mice and humans, and the extensive amount of work that has already been performed and published using mice.

Choice of life stages. The thrombosis, thromboembolism and atherosclerosis protocols will be performed on mice that are normally 8 weeks or older, and at least not younger than 6 weeks of age (minimum age limit).

Typically, what will be done to an animal used in your project?

Mice will be bred with specific mutations in blood clotting factors. These mutations are well supported for normal life and do not cause the mice any specific harm or suffering, but change pathophysiological responses when diseases of thrombosis, thromboembolism and atherosclerosis are triggered. This enables the discovery of new disease mechanisms that may help to develop new treatments for the disease.

The protocols used for triggering thrombosis, involve surgical exposure of blood vessels and application of thrombosis triggering agents or ligation of the blood vessel. This procedure normally lasts no longer than 30-60 minutes. The procedure is either terminal under full anaesthesia, or immediately followed up by surgery to close the site of application and pain relief where needed. In animals that are followed up in time, follow up will last no longer than 2 weeks and more often less than 48 hours.

For the atherosclerosis protocol, mice will be fed an atherogenic (high fat) diet to induce atherosclerosis ("furring up") of the main artery (aorta). This procedure will last up to 20 weeks. At the end of the procedure, the aorta will be taken out for observation in a terminal procedure under full anaesthesia.

Substances may need to be administered (e.g. fluorescent markers for imaging of clots, specific inhibitors, drugs or inflammatory markers) during or before above mentioned procedures. This may occur through injection, oral adminstration or using a portable minipump. No more than 4 substances at any one given time will be administered, and all substances and their administration procedures are well tolerated with minimal discomfort.

What are the expected impacts and/or adverse effects for the animals during your project?

Some of the procedures the mice will undergo during this project will have no or minimal (mild) adverse effects. This is because the breeding of the genetically modified mice does not incur any adverse effects because the mutations are well tolerated unless challenged, and because some procedures are performed under full terminal anaesthesia.

Some of the procedures may incur some discomfort that ranges from minimal or mild to moderate. Pain incurred by surgery in recovery experiments will be minimised using pain relief. In experiments based on thrombosis affecting cerebral blood circulation, cognitive, behavioural or motor skills may be impaired. Mild discomfort will be allowed for up to 2 weeks with careful monitoring and appropriate pain relief. Should any animal show signs of deviation from normal health (piloerection, hunched posture, inactivity or inappetence, >15% weight loss) the experiment will be terminated immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Up to 20% of the mice may show signs of mild severity discomfort (1500 from P1), and up to 80% may show signs of moderate severity (1000 from P2, 1000 from P3, 1000 from P4, 500 from P5).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are three key reasons why the aims of the project cannot be achieved without animal studies:

At present, there are no suitable laboratory alternatives that are able to replace our animal studies, as the studies require viable intact tissues, blood vessels and natural blood circulation, which currently cannot be cultivated or obtained using tissue culture.

Mice are a good model for pre-clinical studies of thrombosis, atherosclerosis and thromboembolism, because basic mechanisms regulating venous thrombosis and other vascular pathologies are similar in mice and humans.

Genetically modified mice allow for targeted and precise testing of the role of certain genes, cells and coagulation factors in thrombosis, thromboembolism and atherosclerosis.

In all experiments, in which the experimental results can be obtained without involving animals, in vitro approaches will be employed. Prior to starting animal experimentation, a comprehensive literature search will be undertaken to ensure that the factor under study is indeed very likely to be involved and also to exclude the possibility that a similar study has already been published. The murine studies will be complemented with and compared to clot structure studies using thrombi obtained from patients after thrombectomy, where the mechanisms leading to thromboembolism cannot be determined.

Which non-animal alternatives did you consider for use in this project?

Currently there are no suitable laboratory methods that fully replicate and can therefore replace the complex interactions between the vessel wall and the blood in determining thrombosis. However, two main technical approaches will be taken that in part replace immediate animal studies, reduce the number of animals needed and underpin the studies proposed in this project licence.

Mutagenesis studies of (generation of mutations in) recombinantly expressed human coagulation factors to test their functional effects using laboratory experiments before introducing the same or similar mutations in the genetically modified mice strains. Using this in vitro approach achieves two main objectives a) similar functional effects can be observed in the mice due to the large degree of evolutionary conservation of the clotting factors between humans and mice, and b) mutations to be introduced will be well tolerated in the mice.

Laboratory flow microsystems will be established that in part replicate the vascular biology of clot formation under flow and used first, before seeking confirmation of the findings in an animal model with the full complexity of the vasculature and the flowing blood.

Why were they not suitable?

Human recombinant protein and mutagenesis technique is very useful to test specific hypotheses about the effects of particular mutations on protein structure and function, and

while they underpin the studies using animals proposed in this project licence, they cannot test specific hypotheses regarding the role of these proteins and mutations in disease.

Laboratory flow microsystems are very useful to test specific hypotheses regarding the interplay of flow and clot formation, but they are not able to test specific hypotheses regarding the role of this interaction in disease development, progression and outcome.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice needed for the studies have been estimated based on 1) prior data and the variation in this data using similar mouse studies, 2) prior data and the variation in this data using laboratory methods directly underpinning the studies, and 3) statistical methods to estimate the minimal number of mice needed to test the hypotheses (objectives) of each part of the studies, based on the size of the effect observed and the variation in the data, with a particular degree of certainty (normally >95%).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Following steps were taken in experimental design to reduce the number of mice to be studies in this project to the absolute minimum: 1) laboratory experimentation wherever possible to replace or underpin any animal study, 2) a high level of expertise and skill of operators involved in the animal studies, 3) statistical methods to estimate the minimal number of animals required to test the primary hypotheses with >95% certainty, 4) using one animal at a time to undertake several measurements (e.g. thrombosis induced in separate blood vessels in the same animal) where possible and acceptable and in terminal procedures under full anaesthesia only, 5) avoiding any possible repeats of previously published literature, and 6) obtaining as many tissue and vessel samples as possible from all animals for laboratory testing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A number of measures will be employed to optimise and reduce the number of animals included in the studies. This includes but is not limited to: 1) efficient breeding strategies where possible and safe to reduce the number of breeding pairs needed to achieve the required numbers, while being mindful of avoiding possible inbreeding and its effects, 2) laboratory studies using recombinant (laboratory) human proteins and flow microsystems

instead of animal studies where possible, 3) efficient use of all tissues and blood of all animals that are included and reach the end of the study, sharing this amongst researchers involved in this project licence and beyond, and 4) use and constant review of statistical methods to ensure that optimal animal numbers are being studied to be able to test the hypothesis (objective) with a reasonable degree of certainty, taking into account the level and variation of the effect observed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The development of blood clots in mice will be studied. The blood clots will be triggered using established methods (e.g. chemical triggers, blood flow reduction) in main blood vessels in the animal (e.g. vena cava, femoral vein or artery, carotid artery). About half of the experiments using chemical injury will be performed under general anaesthesia and

the animals will not wake up at the end of these experiments, so they suffer minimal harm. Other experiments will involve recovery for up to 2 weeks after thrombosis. The model involves careful surgery to minimise suffering and the animals normally recover without any major adverse effects or distress. Any animal showing signs of more than minimal pain or distress will be humanely terminated.

Why can't you use animals that are less sentient?

The blood vessels in which thrombosis is triggered will need to be fully developed and therefore immature animals cannot be used. About half of the experiments are performed under terminal general anaesthesia. The experiments that involve recovery are required to study thrombosis that develops more slowly over time and is therefore more similar to thrombosis in patients with deep vein thrombosis, and to study the role of clot architecture in thromboembolism (e.g. pulmonary embolism and stroke).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The surgical skills and post-operative care of the animals, including pain relief are state-of-the-art, and similar to those experienced by patients undergoing vascular interventions. Where potential situations arise that animals show signs of pain and distress after the procedure that exceed minor levels of harm, monitoring and pain-relief will be increased. In

case any situation occurs of more than the scheduled level of discomfort, animals will be euthanised immediately.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance published on the LASA, LAVA and NC3Rs websites will be followed for research and testing using animals. Guidelines will be reviewed on a regular basis including guidance from the facility's veterinary doctor. With regards to field-specific developments in vascular and blood clotting animal models, guideline documents published by the Models of Thrombosis and Haemostasis Subcommittee of the International Society of Thrombosis and Haemostasis will be consulted. Implication of any developments will first be discussed with the facility veterinary doctor, and subject to approval of protocol amendments by the Home Office.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in the 3Rs will be followed through regular (annual) refresher courses and through communications with the veterinary doctor in charge of the facilities. For the implementation of any advances in 3Rs, protocols in use will be reviewed with the personal licence holders on the project and appropriate modifications will be made where necessary. Any such modification will first be checked with and approved by the Home Office and with the facility's veterinary doctor before implementation.

14. Centrosomes and cilia in development and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cilia, centrosomes, development, neuroscience

Animal types	Life stages
Zebra fish	embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand how centrosomes and cilia contribute to vertebrate development, with an emphasis on neural development, and to model neurodegeneration, seen in Parkinson's and other diseases, in fish embryos.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cilia are hair-like structures on the surface of cells, for example the airway where they sweep up dirt from the air we breathe in. Most cilia in the body act as signalling masts, housing the cellular receptors- antennae - that pick up signals cells use to communicate with each other in backboned animals like humans. This communication is affected in a number of human diseases so it is important to find out how cilia are made and function to understand what goes wrong in these diseases, like polycystic kidney disease. Cilia may

also be affected and involved in the progression of neurodegenerative diseases like Parkinson's. This may offer a route to diagnose this disease but first it must be found out how cilia are affected in this disease.

What outputs do you think you will see at the end of this project?

This project will advance basic science, adding to fundamental understanding of the processes that go on in cells and the body. It will generate new perspectives on important diseases. This information will be published in peer-reviewed scientific publications.

Who or what will benefit from these outputs, and how?

The immediate benefit, during the licence or shortly afterwards, will be to fellow scientists investigating these problems. The new knowledge gained will change the experiments and projects they undertake. The longer term benefit - 10 years or more will be to patients with diseases in which cilia are involved. Knowledge generated by this project could give rise to new diagnostic tests for Parkinson’s. For other diseases that involve cilia, the knowledge may lead to the development of new treatments. Such diagnostic tests or treatments are not part of this project so would require further research and development by commercial entities.

How will you look to maximise the outputs of this work?

We will be collaborating with other research groups, nationally and internationally. We will publish our research in peer-reviewed scientific journals. Involvement of collaborators at these institutions will aid widespread dissemination of the work.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 640

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

One of the main advantages of using zebrafish is that their eggs develop fast and are see-through. This means we can see the results of our experiments quickly. For this project, zebrafish are excellent as cilia are also widespread in all relevant tissues and organs as they develop. Because we will be using eggs for most of our experiments and observations, will be doing few procedures on adult fish. We will need to do some biopsies to test their genetics but otherwise, the fish will lead full lives, breeding to lay eggs that we then study.

Typically, what will be done to an animal used in your project?

We will be creating lines of zebrafish that carry certain mutations, certain genes inactivated or replaced with altered versions. This is achieved by CRISPR using freshly laid eggs. When these eggs hatch and grow into adults, small biopsies from the fins are taken so we can test the genetic status of the fish. These lines of fish will be carriers of the mutations so will not suffer from the effects of inactivated genes. The fish will then be bred to generate the eggs that are studied.

What are the expected impacts and/or adverse effects for the animals during your project?

There will be mild pain associated with the fin biopsies, during recovery as the procedure is done under anaesthesia. This may last one day during initial wound healing. The fin regrows back to its original shape and size.

During fish breeding, there is some stress associated with zebrafish mating and sometime damage to the scales and gills if the adult fish are aggressive. Breeding lasts for one hour; fish are then separated.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For fin biopsies, the severity is expected to be mild for all fish. For breeding, severity is expected to be sub-threshold for 90% of pairings.

What will happen to animals at the end of this project?

Used in other projects
Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals as cells that are grown outside of an animal do not sufficiently resemble those in an animal for us to fully learn how they behave and are affected by different genes and mutations.

How cells behave is strongly affected by the cells neighbouring them. Many tissues and organs are complex, finely detailed structures with many different types of cells. For the tissues we wish to study we cannot yet make these structures in a test tube so we have to use animals. Other animals, such as insects, have different arrangements of cells in some

organs e.g. the brain or lack some structures e.g. the backbone so using them is not an option either.

Which non-animal alternatives did you consider for use in this project?

We do use cultured fish and human cells in our laboratory and we will use them for some aspects of this project.

Why were they not suitable?

These cells do not form the complex structures found in animal tissues and organs which also consist of multiple cell types in close association. We therefore cannot investigate how cilia are involved in the development of these organs or tissues using uniform cells grown in a single layer in a Petri dish.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on the number of lines of fish we will need for this study and the minimum, viable population size for each line.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of adult fish we require is set by the minimum number to keep a line going as a viable population. A pair of fish can lay 100 eggs each week and since we are using the eggs in experiments, this is more than enough material for this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have and will continue to optimise breeding conditions so that we do not have to set up large numbers of pairings for fish breeding to occur. We usually set up six pairs of fish to guarantee laying of the 100 eggs we need for each experiment we perform.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Zebrafish lay eggs so the fish embryos develop outside of the mother. This means that we can see the effects on the embryos without doing anything to the mother or causing harm to any fish fry. Zebrafish eggs and embryos below 5 days old are not protected species under the Animals (Scientific Procedures) Act. The only two procedures the parent fish are put through is being allowed to breed, which is a natural behaviour, and generating lines with altered genes, which involves altering the DNA of the embryos, then monitoring as they develop. In the latter case, we only change one copy of the gene. With the other copy still normal, these fish do not suffer from any effects as one copy of the gene is enough for the cells and tissues to carry on working properly.

Why can't you use animals that are less sentient?

We will be using immature life stages for these studies but to generate embryos that lack certain or have certain genes replaced with altered versions genes, we need parents that have one copy altered. They need to breed to lay the eggs we study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The main procedures we are using are breeding fish and generating new lines. We will refine breeding by using new breeding tanks and ancillary equipment as they become available from manufacturers.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The ZFIN website (based at the University of Oregon) and ZIRC (an NIH funded facility) are well- established sources on the best practice for zebrafish husbandry including breeding and generating and maintaining new lines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will check the NC3Rs website on a regular, monthly basis for new information about how to implement changes to 3Rs practice in our setting.

I will have regular, quarterly discussions with fellow zebrafish researchers to ensure we also put into practice new methods relevant to 3Rs.

15. Neuronal recording on GA Aged Animals

Project duration

0 years 2 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Aging, Synapse, Calcium homeostasis, Memory

Animal types	Life stages
Mice	aged, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To complete an experiment that commenced on a previous project license. To understand the effects of a chronic endocannabinoid treatment on the synaptic and cognitive properties of healthy ageing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The average age of the general population is increasing, by 2030 it is predicted that 20% of the UK population will be over the age of 65. A lengthened life expectancy is often accompanied by a decline in cognitive function in healthy aged individuals. Impairments in cognitive function, such as memory, result in challenges to everyday life in the elderly population and hence reduces their quality of life. Understanding how the aged brain differs from the adult brain will help us identify pharmaceutical targets which may help

alleviate the cognitive decline. This will have the consequence of improving the quality of life of the ageing population and helping to reduce the societal impact that ageing has.

The age-related cognitive decline was formerly investigated in mice under a previous project license and aged mice exhibited cognitive deficits in hippocampal dependent behavioural tasks and changes in hippocampal physiology. The cognitive deficits thus are likely to be linked to the observed changes in hippocampal physiology. Manipulation of the endocannabinoid system has been linked with restoring cognitive function in aged mice. However the mechanisms of how this is achieved is not fully understood. We suggest that this may be due to reversing age-related hippocampal deficits. To address this, under a previous project license, aged animals were treated with an endocannabinoid agonist and completed a series of hippocampal behavioural tasks and then hippocampal tissue was extracted for recordings. The 17 mice put forward for the current PPL have already undergone chronic endocannabinoid treatment under the previous PPL and now need to reach their scientific endpoint by completing a series of behavioural tasks and their tissue collected to measure pre-synaptic calcium. The data collected from the mice in this PPL will be combined with data collected from the previous PPL to to form a complete study.

What outputs do you think you will see at the end of this project?

The project will provide information on alterations in brain function and related behavioural deficits due to ageing. The data will comprise electrophysiological recordings and measures of behavioural performance in aged mice and controls. Initially the work will be written up as a PhD thesis which will be available via publicly searchable databases. The work may also result in a published journal article (subject to discussion with the previous PPL holder).

Who or what will benefit from these outputs, and how?

The translational potential of this work is likely to be realized in the medium to long term. By investigating brain mechanisms underlying age-related cognitive decline the current study has the potential to aide pharmaceutical development of cognitive enhancers for the aged, promote an awareness of age-related mental health issues and improve differential diagnosis for symptom clusters not specific to age, The beneficiaries thus will be clinicians, pharmaceutical researchers and the general public. The immediate short-term beneficiaries will be the scientific community working in the field of cognitive neuroscience and neurophysiology whose research will be informed by our findings.

How will you look to maximise the outputs of this work?

The PhD thesis will become immediately available (timeframe < 1year). In the thesis, the outcomes will be published and described in detail regardless of whether the approach is successful or not.

Species and numbers of animals expected to be used

- Mice: 17

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice represent the lowest species of sentient animals suitable for this work. They are widely used for this type of work and so the results we produce will build upon a substantial body of existing information both from our laboratory and others. We wish to examine how the signalling properties of excitable cells in the brain change with age and how these changes impact on the ability of the brain to learn behaviours that are known to decline with age. We therefore need to use young adult animals and those that have aged naturally. Further the work under this project was started under a different project and needs to be completed in the same animals

Typically, what will be done to an animal used in your project?

Mice will undergo a series of non-regulated behavioural testing and then will be humanely killed by a Schedule 1 method to obtain hippocampal tissue for ex vivo experiments.

What are the expected impacts and/or adverse effects for the animals during your project?

Most mice age normally but as animals get older, they are more susceptible to conditions that are associated with old age. This might include the appearance of tumours or a general deterioration of the condition of the animal. Based on experience with previously tested animals, no adverse effects are expected from behavioural testing and no delayed adverse effects due to previous endocanabinoid treatment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All aged mice will be used by 20 months of age. At this time point, most mice do not display agerelated adverse effects or only experience mild effects. 20% of aged mice may show moderate agerelated adverse effect, with 80% showing mild or no age-related adverse effects. 100% of non-aged mice are expected to experience sub-threshold severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project will investigate how the physiology of excitable cells within the brain is altered with normal aging. To achieve this goal, we need to be able to examine neuronal function in a realistic and preserved network in animals and tissues that have been aged.

Immortalised cell lines are not suitable for most of this work because they do not form realistic neural networks. Brain slices represent an important model for us because the networks are largely preserved and the slices afford access to single cell recordings, population recordings, easy drug access and modern imaging methods that provide more information than electrophysiology alone. Our approach to this is to use the most relevant model to achieve our aim whilst taking all steps possible to adhere to the principles of the 3Rs. Finally, we have to use animals in this particular experiment as it is a continuation of work using animals.

Which non-animal alternatives did you consider for use in this project?

Related research approaches use immortalised cell lines and mathematical modelling approaches to inform and thereby minimise the use of animals. However, there are not yet available models that can realistically model neuronal activity in relation to behaviour. As the work is a continuation of a project involving mice it needs to be completed using animals.

Why were they not suitable?

Non-animal models are not yet sophisticate enough to replace the experiments detailed in this project. They are very useful for helping to predict the actions of drugs, for example, on relatively simple systems and this informs further work using animals. However, models are limited by the information available and animal experimentation helps to provide that information. The work proposed here is a continuation of work carried out in mice so will need to be carried out in the same species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experiments performed in this project are a continuation of a larger study from a previous PPL. The data collected from the estimated number of animals under this PPL (17 mice) will be combined with data collected from a previous PPL (18 mice) to ensure sufficient animal numbers. This estimated number of animals for the overall study is based on published work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In general, we use the NC3R's Experimental Design Assistant tool when designing experiments in our laboratory. This allows us to check the statistical analyses and number of animals to use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible we will tissue share within our facility and other colleagues.
A single brain can yield at least 10 hippocampal slices, 5 cerebellar slices and 3 slices of brain stem, for example.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Calcium sensor selectively expressed at presynaptic terminals in the brain.

This mouse line shows no harmful phenotype. It has been used to examine the nature of synaptic transmission in the hippocampus and cerebellum and most recently to show that during the ageing process, calcium levels in the presynaptic terminals becomes disrupted leading to changes in the release properties of synapses and consequently changes in transmission strength and in the ability of synapses to change their strength. Like wildtypes, when aged, this mouse line shows a reduced ability to learn in behavioural tasks associated particularly with spatial navigation that involves hippocampal integrity. By expressing this sensor in mice, it is possible to prepare brain slices from these animals and record, using optical techniques, directly from the tissue without the need for complex electrophysiology or injection of dyes or other agents to measure neuronal activity. This greatly increases the success rate of experiments and allows more experiments to be performed from a single animal, thereby addressing the 3Rs directly. The use of in vitro tissue allows animals to be used with minimal suffering.

Ageing is a natural phenomenon. If animals start to show adverse effects, then they are treated and if that is not successful, then they are humanely killed so they do not suffer. Whilst animals age, they are well cared for by the staff at the facility, observed regularly and provided with food and water and in cage 'enrichment.

Why can't you use animals that are less sentient?

Mice are mammals and their brain structure is similar to that of humans and they undergo and perform behaviours that are reminiscent of those that humans carry out. It is possible to use less sentient species for some experiments involving behaviour but the availability of transgenic mouse models and the similarity of their brains to humans make them a realistic model for aging and age-related diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Only non-invasive behaviour studies will be performed under this PPL. No food or water restriction will be used, and animals will usually be given positive reinforcement of treats after behaviour testing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to experimentation, the researchers have conducted a systematic review of the literature to ensure that the experimentation is necessary and to ensure that the approach we use is likely to yield the expected results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

1. We undergo regular professional development which includes ensuring that we remain aware of the principles of the 3Rs. We will discuss our work with the named persons, keep abreast of developments through NC3Rs and take advantage of advances in relevant technologies.

16. Infectious disease in pigs and ruminants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Pigs, Ruminants, Infectious disease, Prevention, Pathogenicity

Animal types	Life stages
Cattle	juvenile, adult
Sheep	juvenile, adult
Goats	juvenile, adult

Animal types	Life stages
Pigs	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to investigate disease causing mechanisms of micro-organisms, single or as coinfection, associated with general, enteric or respiratory symptoms in pigs and ruminants of different breeds, their prevention via vaccines or treatments, their detection via novel diagnostic tools and their distribution under field conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The outcomes of this project are a better understanding of infectious pig/ruminant diseases which ultimately will lead to improved intervention strategies such as vaccination or changes in husbandry. Pigs and ruminants are investigated as frequently they can be infected with the same pathogens and they also are reared often in close proximity to each other. For example Erysipelothrix rhusiopathiae is a major pig pathogen and also occurs in lambs and in cattle. Similarly, another pathogen we work with, the zoonotic Hepatitis E virus (HEV) occurs in pigs and ruminants. Concurrent infection of farm animals with two or more micro-organisms (viruses, bacteria) is a common scenario and often increases clinical signs. Knowing which micro-organism interact will help to add in decisions on reduction or even eliminate certain pathogens. Timely demonstration of diseases is important for pig herds in order to implement correct strategies. Closely monitoring field pathogens is important as even minimal changes in the pathogen genome sometimes are associated with increased severity or lack of vaccine efficacy. Earlier detection of such changes will help determine consequences and prevention at a time when the effect on the pig population is still marginal. Finally, improving diagnostic tools and establishing new diagnostic tools for early pathogen detection are needed to monitor disease trends and pathogen spread. Having the correct tools available can be critical to prevent rapid pathogen transmission when emerging pathogens enter the UK pig population or new pathogen subtypes appear.

Pigs and ruminants harbor a variety of coronaviruses (CoV) including bovine coronavirus (BCoV), porcine respiratory CoV (PRCV) and transmissible gastroenteritis virus (TGEV). This research will also examine the susceptibility of pigs/ruminants to infection by emerging pathogens, including those that pose a zoonotic risk, e.g., SARS-CoV-2 but also HEV. Understanding whether farm animal species can be a reservoir or intermediate host for such pathogens will provide benefits for public health. For instance, if ruminants can be infected with SARS-CoV-2 and support virus replication, infected ruminants (including their products such as dairy or meat) pose a reservoir for human infections and will need to be assessed further. Also, if the virus is actively able to replicate in ruminants (=a non-human host), viral genome changes will likely be introduced and it is possible that the virus may become more pathogenic for ruminants as well as for humans.

Lawsonia intracellularis, a major pig pathogen that occurs in many other species including ruminants (deer) and is associated with reduced weight gain and enteric disease in affected species, is causing major economic losses to producers, current vaccines are not always effective, often resulting in antibiotic usage. Similarly, Clostridium perfringens also causes diarrhea but in very young animals wihthout interventional tools that can be used. Novel intervention strategies for enteric diseases in livestock are needed.

Overall, this research directly benefits pigs/ruminants and in the long run will result in healthier pigs/ruminants thereby reducing usage of antibiotics and contributing to pig welfare, a healthier environment and better meat quality.

What outputs do you think you will see at the end of this project?

- Novel information on basic pathogen-host interactions and mechanisms of pathogen pathogenicity will be generated.
- Novel information on basis of intraspecies transmission for HEV and E. rhusiopathiae (ruminant, pigs) and the consequences for the pathogens and host species. This can results in novel cross- species vaccines that can be used and offer protection to variety of animal species.
- Novel information on infectious pathogens such as vaccine targets for future vaccine research will become available.
- Peer-reviewed publications on the investigated topics and outcomes will made available to other researchers but also the general public via open access.
- Possible products of this research include novel vaccines for the enteric pig pathogen Lawsonia intracellularis (L. intracellularis), novel treatment for the systemic and respiratory pig pathogen Porcine reproductive and respiratory and syndrome virus (PRRSV) that could complement current vaccines, and potential novel animal models for future study of mechanisms and intervention strategies for human Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections.

Who or what will benefit from these outputs, and how?

The benefits of this project involve pigs and ruminants, pig and ruminant farmers, meat eaters, veterinarians and scientists at different levels and are summarized below.

Pigs/ruminants

This research directly benefits pigs/ruminants in the long term in several ways. By investigating the consequences of important pathogens on a pig/ruminant and by identifying and testing control measurements against these pathogens, and the possibility of cross-species transmission, this project in the long run will result in controlling diseases

and ultimately healthier pigs/ruminants. Improved diagnostic tools will lead to earlier diagnosis and more timely treatment of pigs which will go beyond the course of this licence. Improving diagnostic tools and establishing new diagnostic tools for early pathogen detection are needed to monitor disease trends and pathogen spread. Having the correct tools available can be critical to prevent rapid pathogen transmission when emerging pathogens enter the UK pig population or new pathogen subtypes appear.

Meat eaters

This research will reduce usage of antimicrobials and contributing to pig welfare, a healthier environment and better meat quality which all is important to people eating the product of the pigs, pork, and living close-by pig farms. Previously, porcine circovirus (PCV2) vaccinated pigs needed significantly less antimicrobial treatment compared to unvaccinated pigs as the general health status in vaccinated pigs was much higher

Veterinarians

Accurate and timely diagnosis of diseases is important for veterinarians in order to implement correct strategies; development of more rapid or multiplex testing strategies can improve therapeutic approach and mitigate disease losses.

Scientists

This research will provide a better long term understanding of the pathogenesis of infectious livestock diseases which ultimately will lead to improved intervention strategies such as vaccination or changes in pig and ruminant husbandry. Closely monitoring field pathogens as part of surveillance studies is important for scientists, veterinarians and diagnostic units as even minimal changes in the pathogen genome sometimes are associated with increased severity or lack of vaccine efficacy or easier interspecies transmission. Moreover, changes in the genome can affect the ability to detect the pathogen. Earlier detection of such changes will help determine consequences and prevention at a time when the effect on the pig population is still marginal. Publications that become available during the course of this project will also contribute to scientific knowledge immediately.

General public

This research will examine the susceptibility of pigs/ruminants to infection by emerging pathogens, including those that pose a zoonotic risk, e.g., HEV, E. rhusiopathiae and SARS-CoV-2. Understanding whether these animal species can be a reservoir or intermediate host for such pathogens will provide benefits for public health. For instance, if ruminants can be infected with SARS-CoV-2 and replicate the virus, infected ruminants (including their products such as dairy or meat) pose a reservoir for human infections and will need to be assessed further. Also, if the virus is actively able to replicate in ruminants (=a non-human host), viral genome changes will likely be introduced and it is possible that the virus may then become more pathogenic for ruminants as well as for humans. Another

virus that needs to be monitored closely as it frequently enters the human food chain via at the abattoir contaminated meat or via pig manure contaminated strawberries and others. Similarly, for bacteria such as E. rhusiopathiae, cattle put in fields or building previously occupied by pigs have a greater risk to acquire infection with this pathogen as we have demonstrated.

How will you look to maximise the outputs of this work?

The outputs of this work will be maximized by collaborations with other investigators within the University but also across other UK institutions and other nations. Any new finding will be presented at farm animal producer and/or veterinarian meetings at the UK, EU and international levels. Knowledge obtained in this research, regardless of success, will be published in international journals and presented at international meetings as we have done in the past.

Species and numbers of animals expected to be used

- Cattle: 130
- Sheep: 130
- Goats: 130
- Pigs: 130

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pigs and ruminants are being used as they represent the natural host for most of the investigated pathogens.

Life Stages

Pigs

- Juvenile/weaned pigs: Most commonly pigs will be used at weaning or shortly afterwards as vaccines or treatments are often given to young pigs around this time. Furthermore, at weaning pigs are often leaving their farm of origin and are co-mingled with pigs from other sites.
  Comingling frequently results in transmission of pathogens between pigs.

- Neonates: When investigating early diarrhea complexes such as seen with Clostridium difficile, pigs need to be used shortly after birth as Clostridium difficile targets suckling pigs and will cause most damage within the first 3-5 days of life. The biology of this bacterium and intervention strategies cannot be tested at later points as pigs are no

longer susceptible. In an upcoming work we will investigate the microbiome associated with C. difficile infection to develop a tool based on feeding probiotics to young pigs at risk to prevent C. difficile infection and lesions. This can only be studied during the time lesions would normally occur.

- Adults: Occasionally breeding pigs will be used as some vaccines are given to sows to protect pigs in the first 3-8 weeks of life via colostrum antibodies.

Ruminants

- Juveniles: Young animals at 4-6 weeks of age will be used. Ruminants at this age are easier to house and handle and the age is suitable for COVID-19 research.

Typically, what will be done to an animal used in your project?

For typical investigations on conventional sourced pigs/ruminants, they will be obtained from a commercial farm at weaning age, will be randomly divided into several groups and rooms, and after an appropriate acclimation time may be vaccinated against the pathogen under study in the research facility with a re-vaccination 2-4 weeks later if needed. Subsequently after approximately 4-6 weeks after initial vaccination, the animals will be challenged with the pathogen of interest. After vaccination and challenge samples will be collected on a regular basis to determine if the pathogen is present and if an immune response against the pathogen can be detected. Samples that may be collected include blood samples, nasal swabs and/or rectal swabs among others. The various sample types may be tested by PCR to confirm presence and amount of the pathogen, by ELISA for antibodies, or by other tests for cytokine presence and others. At termination of the project the animals will be killed using a schedule 1 method. This may be followed by a necropsy were lesions in the animals are assessed and additional tissues may be collected.

What are the expected impacts and/or adverse effects for the animals during your project?

Mechanism studies and vaccine trials

Expected adverse effects:

- Lumps or redness at the vaccination site. The duration of these adverse effects may last up to 2-3 days.

- Haemorrhage during blood collection. The duration of the direct impact of hemorrhage on the pig is commonly 10-20 min but the pig may be weak for up to 3 h.

- Clinical signs after challenge: Fever, diarrhoea, weight loss, respiratory signs, lethargy. The duration of these clinical signs depends on the pathogen invovled but typically clinical signs may start as early as 1-2 days after challenge for enteric pathogens such

as C. difficile or as late as 5-7 days for respiratory pathogens such as PRRSV.

Control sample derivation for test development (POLE)

Expected adverse effects:

Haemorrhage during blood collection. The duration of the direct impact of hemorrhage on the pig is commonly 10-20 min but the pig may be weak for up to 3 h.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mechanism studies and vaccine trials

Likely/expected level of severity: Mild-to-Moderate. At termination the pigs/ruminants will be killed using a schedule 1 method.

Pigs: 30% moderate, 70% mild

Ruminants: 10% moderate, 90% mild

Control sample derivation for test development (POLE)

Likely/expected level of severity: Mild. At termination the pigs will be rehomed.

Pigs: 100% mild

Ruminants: 100% mild

What will happen to animals at the end of this project?

- Killed
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are working with the target species and will investigate diseases and intervention strategies in pigs and ruminants. Studying the disease in the natural host is essential in order to inform management and production decisions which are immediately translatable. In addition, we will also develop and validate diagnostic tools important to detect pathogen

presence on livestock farms. For this we need access to known positive and negative control samples which ideally would come in from the target species.

Besides pathogens already present in UK livestock, this research will also examine the susceptibility of pigs/ruminants to infection by emerging pathogens, including those that pose a zoonotic risk, e.g., SARS-CoV-2. Understanding whether these animal species can be a reservoir or intermediate host for such pathogens will provide benefits for public health. For instance, if ruminants can be infected with SARS-CoV-2 and support virus replication, infected ruminants (including their products such as dairy or meat) pose a reservoir for human infections and will need to be assessed further. Also, if the virus is actively able to replicate in ruminants (=a non-human host), viral genome changes will likely be introduced and it is possible that the virus may become more pathogenic for ruminants as well as for humans.

Which non-animal alternatives did you consider for use in this project?

Non-animal derived alternatives including cell lines, organoids, tissue sections have been identified and will be used whenever possible to generate data.

Why were they not suitable?

It is not feasible to quantify organ colonisation, systemic spread, tropism or immune responses mediating pathology or protection solely in cell-based assays or organ culture systems such as lung or liver slice cultures. Non-animal derived alternatives as listed above, are not suitable to study the development of a disease, infectivity of a certain pathogen, determining if vaccines protect an animal and generation of positive controls for diagnostic tests can only be done in live animals. Cell culture systems are not suitable to study interactions between the animal's immune system and the disease causing agent. Similarly, antibody responses ("titre development") can only be studied in pigs/ruminants and not in cell culture systems.

The decision to use animals will only be taken once other avenues of research such as in vitro studies have been exhausted. Alternative routes will be considered whenever possible and feasibility of using alternatives will be assessed for each separate study. Cell culture studies are being done and results will inform future animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers that will be used will be based on planned funded experiments and experience doing similar studies in the past.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- The Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) will be used.
- We will check relevant literature on this topic including published studies for the pathogens investigated.
- We will also seek advise from experts in the area of investigation.
- We will also consult with our statistician.
- Regulatory requirements will be followed for PK studies

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Pilot studies will be done whenever no or limited data are available.
- Animal studies that could be shared with other researchers to investigate two scientific questions at the same time will be preferred.
- Computer modeling to investigate aspects such as infectivity speed will be used in the optimization phase for the animal project.
- Tissues and liquids such blood will be shared.
- Whenever feasible biobanks of tissues or blood will be established.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs and ruminants (sheep, goats, calves) are key reservoirs of the investigated infectious agents and are also be susceptible to pathogens circulating in humans including HEV, Erysipelothrix rhusiopathiae or SARS-CoV-2. Disease is not always reproducible in pigs or ruminants compared to other model species; however usually viremia/bacteremia and shedding can be assessed and is useful to assess intervention strategies such as vaccination. Studying the disease in the natural host or a host that is very similar to the natural host (pigs/ruminants and humans) is essential in order to inform management and production decisions which are immediately translatable.

Other groups have reported using rodent models with many of the infectious agents in pigs we commonly use; however, the outcomes vary between different groups, further emphasizing that the models are not robust. Rodent models may be useful for analysis and characterization of immune responses against selected pig pathogens and in the past our group has used a mouse PCV-2 model. We infected three different mouse lines porcine circovirus type 2 (PCV2) but we were not able to show infectivity, seroconversion, disease or lesions in any of the mice. While it is widely accepted that rodents can play a role in transmission of some pig pathogens, rodent infection, lesion development or disease is questionable and these models are not currently useful for the pathogens we work with to study disease development in the target species.

A clinical scoring system refined under preceding studies will be used to monitor the severity of infections and identify animals requiring increased monitoring, treatment or euthanasia. Commonly we measure rectal temperatures in pigs/ruminants to identify animals that develop a higher than expected fever for a prolonged time.

When vaccines or drugs are used, usually these are typically commercially available and licensed products for pigs/ruminants. While injection site reactions are not expected we typically monitor the reaction sites for up to three days for any reactions. With the expectation of caesarian-derived, colostrum-deprived (CDCD) and colostrum-deprived (CD) pigs in the first days of life, all pigs are group housed. The CDCD and CD pigs that will be housed in individual isolators are separated from their litter mates by Plexiglas allowing them to see their siblings. Refined models are currently being used that comes from years of experience and will be further refined in the UK.

For sample collection we often use oral fluid. Oral fluid collection does not interfere with a pig's normal behaviour as not direct contact is necessary to obtain this sample type and therefore this can be considered as refined procedure.

We will share any propagated serum controls to other potential end users (companies, diagnostic units) so that they do not have to create their own control serum in pigs/ruminants.

Why can't you use animals that are less sentient?

Pigs and ruminants are the target species for the pathogens investigated. Lower species such as mice or rats cannot be used.

Pigs and ruminants can be readily infected with E. rhusiopathiae, pigs can be infected with HEV (unknown for cattle but suspected), pigs and deer (not yet determined for cattle, sheep and goats) are reservoirs for L. intracellularis, pigs and ruminants are targets for coronaviruses including PRCV, bovine respiratory coronavirus and also for SARS-CoV-2. The experimental evidence for SARS-CoV-2 infection in ruminants or pigs is not very clear and needs to be further confirmed to rule out that ruminants or pigs act as possible virus reservoirs for humans. It needs to be further investigated to determine whether or not pigs and ruminants can be consistently infected with SARS-CoV-2 as results so far are

conflicting. After experimental inoculation, research groups confirmed antibody development in all pigs without detectable viremia or virus shedding. Other found not evidence of seroconversion, yet others found seroconversion, virus shedding with infections virus being isolated in few pig. Cattle has been shown to be be susceptible to experimental SARS-CoV-2 (virus shedding in nasal secretions) however, lesions and tissue distribution of SARS-CoV-2 was not investigated. It is important to determine if SARS-CoV-2 can also infect sheep and goats and if there are lesions and viable virus in lungs or other tissues for further assessing these species as possible virus reservoir or intermediate host.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals will be closely monitored depending pathogen and intervention. A plan for adverse effects is in place for each pathogen and model. Pain management will be provided if needed.

Refinements in place: Pigs/ruminants will be kept in social groups whenever possible. If this is not possible, pigs/ruminants will be able to see each other through glass partitions. All pig rooms/pens will be equipped with environmental enrichment. Other refinements that are in place include usage of real- time PCR cycle threshold numbers or virus genome calculation to inform endpoints.

A statistician will be consulted when planning individual studies to determine the minimal number of pigs needed to have enough statistical power to determine significance if there are true differences between groups.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the NC3R guideline (https://nc3rs.org.uk/the-3rs)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I continuously inform myself by reading relevant literature on 3Rs (mostly online). I will also contact others including AWERB committee members prior to study start and ask for further advise. I also routinely search databases on published information for studies I am intending to avoid possible duplications.

17. Regulation of gene expression in vertebrate development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Development, Stem cells, Therapy

Animal types	Life stages
Zebra fish	embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to understand the genetic programs needed to create tissues and organs, such as blood, muscle, pancreas and liver, in an embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The ability to make tissues, or even organs, rather than use donated organs, for transplant is a goal of modern medical science. However, currently scientists cannot precisely recreate tissues with 100% efficiency. For instance, during the process some cells may not form the wanted cell type but form another cell type altogether. In other cases, the 3

dimensional structure of the tissue cannot be recreated and the cells do not work efficiently without that structure. In order to overcome these difficulties, it is important and necessary

to study first in detail the embryonic process of making tissues, so as to better recapitulate it.

With a better knowledge of the genetic programs needed to create tissues and organs, we can apply the knowledge to making tissues outside the body, by coaxing stem cells or other cells to use the same programs. Such 'in vitro' generated tissues can then be used to replace damaged tissue in humans or animals after injury or disease. Alternatively, this understanding may lead to ways of rebooting these programs in the damaged tissue so that repair takes place in the patient's body, without the need to generate replacement tissues in vitro.

What outputs do you think you will see at the end of this project?

We expect to have a better understanding of the genetic programs that direct the formation of different tissues or organs, such as muscle, heart and pancreas. This information will be disseminated through open access publication in scientific journals and presentations at scientific conferences. Results will also be highlighted through press releases if appropriate. We will also have generated large datasets that will be deposited in public archives for use by the scientific community.

Who or what will benefit from these outputs, and how?

The beneficiaries of these outputs in both the short and long term will be other academic researchers studying developmental biology, stem cell biology and regenerative medicine. In the longer term, patients will benefit from application of the outputs to regenerative medicine therapies.

How will you look to maximise the outputs of this work?

Any new knowledge will be disseminated via scientific publication, conference papers and depositing datasets in public archives, allowing other scientists to use the data. Where possible we will collaborate with other scientific groups, for instance with joint PhD students, to ensure maximum output.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying how tissues form in an embryo and therefore use embryonic stages. We use the zebrafish embryo for these studies for multiple reasons. The embryos develop in a

simple saline solution outside the mother and so they are easy to observe without needing to perform any procedures on the mother. The embryos are transparent, meaning we are able to visualize internal organs without the need for surgery. When mated, adult zebrafish produce several hundred embryos in one spawning, meaning we have a large amount of material for study, and this limits the number of adult fish we need to keep to ensure we get statistically relevant data. And while fish are not humans, we already know that the early events of embryogenesis used very similar genes and so what we learn in fish is often applicable to humans.

Typically, what will be done to an animal used in your project?

Adult zebrafish used in our project mate to produce embryos, which are what we study. We use very early embryos in our studies, usually before 24 hours of development, which is before a functional nervous system has formed. We keep the adult fish until they are around 18 months old and they mate approximately every two weeks. When appropriate we may alter the DNA of the embryos and grow them to adulthood, and then mate these adult fish to produce genetically altered embryos for studies.

What are the expected impacts and/or adverse effects for the animals during your project?

We may need to take a fin biopsy of adult fish to confirm their genetic status. While this is done with anaesthetic, it is possible that fish may experience transient mild discomfort during the procedure, and in extremely rare cases infection of the biopsy site may occur. We do not expect any adverse effects from the genetic alterations we make, but it is possible that this may happen, e.g. tumours, abnormal behaviour. If we observe adverse effects occurring after fin biopsy or in any genetically altered zebrafish we will euthanise them immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for adult fish that have fin biopsy is mild. Fin biopsies are experienced by ~25% of fish.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to experimentally establish the program of gene expression that controls early development in vertebrate embryos. We need to better characterise these programs so that we can recapitulate them in cells grown outside the body (called in vitro differentiation) for human regenerative medicine therapies and for modelling disease. This is because, although scientists have the ability to make various cells and tissues outside the body, the methods are still not fully efficient. Research suggests recapitulating the program of gene expression used in an embryo can increase the efficiency of this in in vitro differentiation. It is therefore important to establish the detail in the developing embryo.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives include in vitro differentiated stem cells.

Why were they not suitable?

As described above, in vitro differentiated cells do not yet represent a full model of embryonic development, and it is this gap that we seek to fill in this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This number is based on the number of animals that will need to be raised to generate or maintain GA variants of zebrafish. When breeding fish to maintenance a variant genetic alteration we expect, due to Mendelian genetics, that 50% of the fish will carry the variation. This means that for every 50 fish required, we must breed 100. For generating GA fish by transgenesis or genome editing, we find that 10-50% of fish are GA when we test them, depending on the type of genetic alteration. This means that for every 10 fish carrying the required genetic alteration, between 20 and 100 fish must be raised.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The latest technologies for introducing genetic changes in zebrafish will be used to ensure the procedure is as efficient as possible, thereby reducing the number of animals that need to be used. Power calculations were performed to determine the numbers of GA fish needed for experimental analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We keep our zebrafish in a state-of-the-art aquarium that has health monitoring systems to ensure optimal water chemistry and we use tested feeding regimes that maximise the survival and health of fish. This reduces the number of fish that need to be raised to adulthood as we expect very little death in juvenile stages.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish for this project. Adult zebrafish mate to produce embryos, which does not cause the animals harm or distress. Our experiments are performed on the embryos that are less than 5 days old (ie non-protected), indeed we usually use embryos that are less than 24 hours old. We anaesthetise adult fish prior to fin biopsy and take only a small part of the tail fin; the fins regenerate within two weeks and does not cause lasting harm to the animals.

Why can’t you use animals that are less sentient?

The experiments carried out in the project are done on immature life stages, ie embryos less than 5 days old. In order to generate these embryos, we need to use adult fish for spawning.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Zebrafish are monitored for 24-72 hours after fin biopsy to check their recovery, normal swimming behaviour and if there is any infection of the biopsy site. Those that do not recover, which is approximately 1%, are killed by a schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult the most up-to-date scientific evidence for experimental procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We read scientific literature, attend scientific conferences where advances are discussed, and communicate with other local research groups and aquarium staff about the implementation of advances.

18. Role of the inhibitory neurotransmitter naag and its transporter in pathologies involving glutamatergic dysfunction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Metabolism, Diabetes, Glutamate signalling, Schizophrenia, Prostate cancer

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary aim of this project is to delineate the role of glutamatergic signalling in communication pathways responsible for the regulation of metabolism. Our secondary aims are to characterise the role of the inhibitory neuropeptide NAAG in schizophrenia, diabetic retinopathy and prostate cancer, which have, together with type 2 diabetes, been genetically linked to a transporter protein we study in our lab which is involved in glutamate signalling.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity remains one of the major health challenges of modern times. By the start of this millennium, the WHO reported that the number of obese adults increased to over 300 million with 41 million children under the age of five classified as overweight or obese by 2016. Co-morbidities, such as lifestyle- induced type 2 diabetes and cancer, are on the rise in parallel, and the number of individuals with diabetes worldwide has been rising exponentially in the last 40 years.

Studies on the endocrine cells - and the hormones they secrete - which regulate our metabolism, such as insulin-secreting pancreatic cells or hormone-secreting gut cells, have advanced our knowledge of how the human body maintains metabolic homeostasis. However, in recent years it has become increasingly clear that, although studying metabolic systems in isolation is tremendously useful, it will only ever allow us to view a snapshot of a specific metabolic state or system in time. Furthermore, when investigating human metabolic disorders, such as obesity and diabetes, we need to understand how all the physiological systems modulated by metabolic dysregulation are affected. The metabolic research conducted in this study investigates how glutamatergic signalling modulates the cells that orchestrate mammalian metabolism via hormone secretion.

Our lab has studied a particular protein of interest that transports glutamate and glutamate-containing molecules across cell membranes. Studies showed that increased gene promotor activity, which results in the overexpression of this protein, in subcutaneous adipose tissue predisposes humans to increased weight gain and the development of type 2 diabetes with age. However, although overexpression of this protein was metabolically detrimental, it protected individuals against schizophrenia and bipolar disorder. Our lab has found that when this transporter is genetically absent from mice, it results in a lean and metabolically healthier phenotype (which is exactly the opposite of the human overexpression phenotype). Furthermore, our metabolic studies demonstrated that mice lacking this transporter are hyperactive and may present a schizophrenia-like phenotype; this is consistent with findings that a primary substrate of this protein, N-acetylaspartylglutamate (NAAG), which is an inhibitory neurotransmitter, is highly associated with schizophrenia and bipolar disorder.

Furthermore, this transporter has been associated by genome-wide association studies (GWAS) to diabetic retinopathy, a complication of diabetes in which high glucose damages the retina. Glutamate is the primary neurotransmitter in the retina, and NAAG is also present at high levels, supporting the hypothesis that this transporter may play a role in retinal glutamatergic signalling. Several other putative substrates of this transporter play instrumental roles in the molecular mechanisms underlying the development of diabetic retinopathy .

Interestingly, it has also been reported in human genetic studies that our target transporter is upregulated in metastatic prostate cancer. The substrate of our protein, NAAG, is known to serve as an important energy source for many cancers. Several genome-wide association studies (GWAS) linked our target protein to the development of metastatic prostate cancer and suggest that this transporter could play a central role in the mobilisation of energy sources and subsequent tumour growth and metastasis in prostate cancer.

Overall, little is known about the target protein in this study, and our laboratory will therefore, alongside our primary metabolic investigation, also explore its possible roles in diabetic retinopathy, schizophrenia and prostate cancer.

What outputs do you think you will see at the end of this project?

Our primary goal is to shed light on the cellular and molecular mechanisms underlying metabolic regulation and dysfunction, including a primary complication of diabetes, diabetic retinopathy.

Furthermore, we want to delineate the roles of the transporter protein we study in schizophrenia and prostate cancer. We expect our findings to effectuate peer-reviewed publications that will inform general scientific audiences. Given the undeveloped nature of our projects studying schizophrenia and prostate cancer, we expect preliminary results to serve as the base for grant applications to fund the maturation of these research programmes in our lab. All models developed during the research will be freely available to the scientific community.

Who or what will benefit from these outputs, and how?

Short term outcomes include shedding light on the mechanism of glutamatergic signalling in obesity, diabetic retinopathy, schizophrenia, and prostate cancer. Specifically, we will delineate the role of the inhibitory neuropeptide NAAG and its transporter in these pathologies. These findings will constitute basic research immediately valuable to other researchers in the field.

In the long term, our findings will, first and foremost, advance current knowledge about the pathophysiology of obesity and the dysregulation of appetite and energy metabolism in obese patients in order to help pave the way to suitable treatments for these patients. Secondly, our findings will help us understand the role of NAAG in schizophrenia and will shed light on the use of NAAG receptor agonists and antagonists in the clinic. Thirdly, we will explore the possibility of finding new early markers to predict prostate cancer progression in man. Finally, we will determine if this transporter of interest plays a role in diabetic retinopathy.

How will you look to maximise the outputs of this work?

Our laboratory regularly attends national and international meetings where preliminary data and experimental hurdles from the proposed studies will be discussed with the greater scientific community. Project data will be made available to all interested researchers through open access publications. Our laboratory also collaborates with a multinational pharmaceutical company that is a major developer of treatments for diabetes. Models generated during the course of the study will also be made available to the greater scientific community. There is active engagement at both the home establishment and other establishments with interest in the subject, which involves the discussion of career progression, ethical data management, healthy living, and the prevention and treatment of obesity.

Species and numbers of animals expected to be used

- Mice: 15,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Metabolism is a complex and integrative physiologic process involving all organs, notably the gut, pancreas, brain, and fat depots. Scientific research regularly utilises in vitro models of some isolated aspects of this system, such as gut-derived stem cells or model cell lines for gut or pancreatic cell types, but many questions can only be answered in an intact system where the cross-talk between organs is present. Much metabolic signalling occurs through inter-organ communication via hormones, each of which have distinct inducers, targets, and clearance mechanisms. Mouse and human energy metabolism are remarkably similar, and therefore mouse models are the most appropriate model to answer these more complex systemic questions. As our work focuses on adult metabolism and the development of dysregulated metabolic processes over time, adults will be the most prominent life stage we use in our studies. Occasionally, juveniles will be used when a procedure takes significant time to take effect e.g., the high fat diet induction of obesity where juvenile mice are placed on a high fat diet to mimic human life-style induced obesity; but our target experimental outcomes will always come from adult mice.

Typically, what will be done to an animal used in your project?

Most mice in our project will exhibit a metabolic phenotype, which may be genetic or experimentally induced. Therefore, some mice will need no intervention to produce this phenotype (e.g., knockout mice), whereas others will be fed a high fat diet or injected with a drug (e.g., subcutaneous injection of hormone receptor agonists). Blood samples may periodically be needed, and some mice will undergo mild imaging methods (e.g., EchoMRI to assess body composition). Some mice will undergo 2-4 metabolic tolerance tests, which consist of a fast, injection of a metabolite such as glucose or insulin (intraperitoneal or

oral), and several small blood samples. For our studies on diabetic retinopathy, diabetic mice will undergo non-invasive glucose urine testing regularly to determine onset of hyperglycaemia, after which they will have blood glucose testing regularly; they may experience one intravitreal injection for acceleration of retinal pathology.

Another of our studies focuses on schizophrenia and involves either genetic mouse models with a schizophrenic predisposition or drug-induced schizophrenia. The latter may receive a schizophrenia- inducing drug such as PCP for up to 2 weeks via subcutaneous minipump implantation. All mice in this line of study would undergo non-aversive behavioural testing (e.g., Y-maze, auditory pre-pulse inhibition). A minority of mice we use will instead be genetic models of prostate cancer who will only experience blood sampling and will be euthanised prior to developments of any metastases or cancer complications, such as organ failure.

What are the expected impacts and/or adverse effects for the animals during your project?

A huge majority of our studies have few adverse effects and do not produce long-lasting harm. Mice may experience several instances of substance administration and a finite number of blood withdrawal procedures, none of which causes more than momentary discomfort. complications. Our metabolic studies focus more on obesity than diabetes pathology, so diabetic mouse models will be avoided for these studies. However, the study of diabetic retinopathy necessitates underlying diabetic pathology. To minimise suffering of diabetic mice, they will be killed as early in disease onset as possible to address our scientific outcomes, usually within 3 weeks of glycosuria detection. After glycosuria is detected, blood glucose will be measured to ensure excessive levels are not reached. Our schizophrenia studies are expected to induce abnormal behaviour, but no physical pain or suffering. Mice modelling prostate cancer will be killed before or during early stages of tumour development and will not develop metastases or other cancer-related complications.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: 80%
Moderate: 20%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Metabolism is a complex physiologic process involving all organs, notably the gut, pancreas, brain, and fat depots. Much metabolic signalling occurs through inter-organ communication via hormones, each of which have distinct inducers, targets, and clearance mechanisms. The metabolic physiology underlying glucose homeostasis and energy balance is well conserved in humans and mice, which is not true for non-mammalian species. Therefore, mouse models are the most appropriate approach by which to answer questions involving whole-system metabolism.

Which non-animal alternatives did you consider for use in this project?

We do (and will continue to do) a significant amount of in vitro work using cells lines, including lines modelling gut cells, pancreatic alpha- or beta-cells, and prostate cancers. We also perform experiments in ex vivo models of gut crypts to answer specific questions about hormone secretion in response to certain metabolic stimuli. After preliminary studies in the eye, ex vivo retinal explants can be used to test new mechanistic hypotheses. All of this work is very important for laying the foundation for animal experiments, in many instances serving as a proof-of-concept.

Why were they not suitable?

These in vitro approaches are limited and cannot be used to fully address the aims set out in this project. In particular, it is impossible to mimic inter-organ communication, especially modulation from the brain. It is also impossible to replicate developmental processes outside of an animal that may be important for underlying mechanisms. Furthermore, overall body weight and metabolic health cannot be assessed in non-animal models, nor can behavioural outcomes associated with schizophrenia.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimated number of mice is derived from our previous studies or, in the case of techniques new to our lab, data reported in the literature but modified to fit our specific experimental needs. We study both males and females as independent groups due the known sex-dependent biological differences in metabolism regulation and schizophrenia.

Not only must we consider our primary procedural goals within this protocol of metabolic tolerance tests and metabolic imaging along with the different mouse models we plan to use, we also do extensive post-mortem analyses (such as western blot and histology) necessitating substantial amounts of tissues. Furthermore, we propose a number of non-aversive behavioural tests that, for the sake of experimental integrity, cannot all be performed on the same mice. Many of our studies (particularly those that are neuro-focused) will be greatly enriched by examining mice at different ages.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experimental design is based on the ARRIVE guidelines, with very clear, measurable outcomes and pre-determined power analyses to ensure optimal usage of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use effective breeding programmes based on existing data to ensure we do not produce excess animals. We will always confirm the viability of any new model or drug for our uses by rigorously analysing the literature. When using new techniques, we will use small pilot studies to not only confirm the efficacy of the method but also to ensure that all of our downstream assays are optimised.

Furthermore, we commonly collect samples from other groups in our department that do not need our organs of interest, e.g., we regularly collect gut samples from a group that is devoted to brain research so that we can optimise new techniques. This helps cut down on the number of mice needed from our own protocols for optimisation-oriented tissue assays. We always strive to utilise as much tissue from one mouse as possible - if there is not immediate use for a tissue, we routinely freeze or otherwise preserve samples for later use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For our obesity studies, we will opt for high fat diet-fed mice or ob/ob mice, neither of which develop overt diabetes or diabetic complications, as those aspects are not currently necessary for our metabolic research programme. We will perform metabolic monitoring of

activity and energy expenditure that will be carried out in a housing environment similar to the animal's home cage but with data collection capabilities. This is more refined than canonical metabolic cage systems with wire-grid floors and absence of bedding and enrichment, which are extremely stressful for mice. We will opt for thermal imaging as opposed to internal (anal) temperature readings, as it is sufficient for our purposes.

To minimise suffering of diabetic mice in our retinal studies, we may use a previously published model variant that includes one intravitreal injection into one or both eyes of a diabetic mouse. This results in a significantly accelerated time course for diabetic retinopathy development, meaning that mice will typically endure hyperglycaemia and the associated complications for approximately three weeks instead of the 12 or more weeks natural development of retinopathy would take. Intravitreal injections will take place with use of a stereomicroscope and stabilised syringe to ensure proper needle placement and to prevent damage to the lens.

To determine diabetes onset, we will test glycosuria, as this is a non-invasive procedure that only involves briefly handling the mouse to induce urination and catching a drop on test strips. This method is a very sensitive indicator of diabetes onset and is much more refined than blood glucose testing.

Following glycosuria detection, mice will regularly have blood glucose tested to ensure they do not reach our humane endpoint

Substance administration of the drugs we plan to chronically administer, such as hormone receptor agonists or drugs that induce schizophrenia-like symptoms, can be refined by using osmotic minipumps in lieu of daily injections. Subcutaneous minipump implantation is a simple procedure that is well- tolerated by the mice. This eliminates the use of needles and significantly decreases the amount mice are handled, which can be stressful. Furthermore, when a drug can be administered via multiple routes, we will always choose the least painful option.

Regarding our schizophrenia models, we have opted for drug-induced models as opposed to those involving maternal separation or hippocampal lesioning which inflict more suffering. Our prostate cancer mouse line(s) will not be kept long enough to experience metastases or complications such as organ failure, as we aim to study the molecular events preceding metastasis.

Why can’t you use animals that are less sentient?

Neither fish nor lower organisms, such as the fly, are suitable for these studies, as it is difficult to investigate metabolism in aquatic organisms, fish are not endothermic and their islets are arranged differently, and the fly is too evolutionarily different. The mouse has well-conserved physiology, including multiorgan control of glucose homeostasis, compared with humans. Conservation at the level of the genome is also high.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our animal procedures are quite simple and are well established. In order to minimise the number of injections that a mouse must undergo, we will utilise osmotic minipumps whenever possible and more refined. The few surgical procedures we present are mild, and standards will be guided by animal welfare resources and the expertise of our vets and NACWOs. When we begin using a drug that is new to our lab, we may conduct a small pilot study with subcutaneous injections versus minipump implantation to assess the scientific viability of minipump use. If we learn of approaches to improve animal welfare or data reliability, we will not hesitate to refine our methods.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the ARRIVE and NC3R guidelines for experimental design as well as best practices recommended by LASA and the Handbook of Laboratory Animal Management and Welfare (2013, Wolfensohn & Lloyd).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institution regularly sends out notices regarding the NC3R's resources and webinars, which we will utilise. We also stay up-to-date on the literature in the field and will observe modifications that can be made to improve our own approaches.

19. Cellular electrophysiology of cardiac arrhythmias

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

antiarrhythmic drug, arrhythmia, cardiac myocyte, ion channel, pacemaking

Animal types	Life stages
Guinea pigs	adult
Rabbits	adult
Mice	adult, juvenile, neonate, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim of this project is to advance understanding of the cellular mechanisms responsible for controlling normal heart rhythm and the alterations that underlie disturbances in rhythm (arrhythmia). It is hoped that this research will lead to the development of novel treatments, or improvements in existing therapies, for the management of arrhythmia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The heartbeat arises through the generation of rhythmic electrical impulses that are conducted throughout the heart, causing it to contract in a co-ordinated manner to pump

blood around the body. Abnormalities in the rhythm of the heartbeat (arrhythmias) are a major cause of death and cardiovascular disease. These arise either through the generation of rogue impulses or conduction abnormalities that alter the propagation of the electrical impulse, either delaying conduction or resulting in circuits of propagation that cause a rapid, irregular and un-coordinated heartbeat. Arrhythmias are often treated using drugs or implanted devices that take over the pacemaking function of the heart.

These approaches have limited effectiveness and many anti-arrhythmic drugs also have unwanted side-effects, including causing an irregular heartbeat. The aim of the outlined studies is to advance understanding of the mechanisms underlying the maintenance of normal heart rhythm and to identify novel approaches to improve the treatment of arrhythmia.

What outputs do you think you will see at the end of this project?

The principal output of the work will be novel information about the cellular mechanisms responsible for the maintenance of normal heartbeat and how alterations in these mechanisms disrupt heart rhythm. In so doing the studies aim to identify novel targets for the treatment of disturbances in heart rhythm.

These findings will be communicated through conference presentations, publications in peer-reviewed scientific journals and via Open Access routes so that the results are available without subscription.

Reports will also be generated for the funding bodies. Where appropriate, results may be communicated to the public through press releases and outreach activities

Who or what will benefit from these outputs, and how?

In the immediate to medium term, we expect the outputs to benefit cardiologists, cardiac surgeons and basic scientists around the world with an interest in the mechanisms responsible for maintaining normal heartbeat and the treatment of arrhythmia. In the long term the findings are expected to contribute to the development of improved treatments to restore normal cardiac rhythm to patient suffering from arrhythmia.

How will you look to maximise the outputs of this work?

In addition to the publication of results in peer-reviewed scientific articles, data will be registered on open access data archives.

Species and numbers of animals expected to be used

- Mice: 2,000
- Rats: 250
- Guinea pigs: 250
- Rabbits: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats and mice have been widely used for studying heart function. As a consequence, extensive background literature exists for these species, making them an appropriate model species to achieve the scientific objectives of many of the outlined studies. In particular, rats are an appropriate model for studying arrhythmias associated with the upper chambers of the heart (atria). Mice are readily amenable to genetic alteration. Consequently, they will be used for studies examining the role of specific genes and proteins in the maintenance of normal heart rhythm. However, neither rats nor mice are suitable for modelling the disturbances to heart rhythm that arise in the ventricles of humans.

Therefore, such studies will utilise rabbits, which have a conduction system that closely replicates that of humans. The ionic mechanisms underlying repolarisation of the rabbit ventricle are closer to those of the human than are those of rat or mouse ventricles. In addition, it is easier to identify the relevant structures in rabbits than in smaller laboratory animal species. Furthermore, the larger size of the rabbit heart means it is also possible to obtain atrial and ventricular heart muscle cells from the same rabbit hearts as used for the study of the conduction system [reduction]. Therefore, in so far as is possible, these hearts will also be used to investigate atrial and ventricular cell electrophysiology for the objectives covered by this project. However, the rabbit has limitations as a model for the human in that it lacks a certain component that is important to human ventricular function. Consequently, some studies will be undertaken using guinea pigs as they have the relevant ventricular component missing in the aforementioned species.

Previous studies have shown that the structure of heart muscle and of heart muscle cells is extremely important to the mechanisms responsible for maintaining normal heartbeat and that disruption of these structures can cause disturbances to heart rhythm. As these structures are immature in hearts from very young animals it will be necessary to use adult animals for the outlined studies.

Typically, what will be done to an animal used in your project?

Guinea pigs, mice, rabbits and rats: Animals may be given an injection that causes no more than mild transient pain shortly before they are killed using a non-Schedule 1 method in order to obtain tissues for ex vivo studies.

Mice: Mice may be bred with genetically alterations relevant to the regulation of heart rhythm. None of these animals are expected to suffer any adverse effects as a result of the

genetic alterations. Small biopsy samples (typically ear punches) will be taken post weaning for the purpose of genotyping. This causes no more than mild transient pain.

What are the expected impacts and/or adverse effects for the animals during your project?
All animals: Some animals may experience mild transient pain when given an injection.

Mice: No adverse effects are expected as a result of the genetic alterations however most will experience mild transient pain as a result of biopsy sampling for the genetic screening.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for all animals is mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to study fully the cellular and sub-cellular mechanisms underlying the maintenance of normal heart rhythm, or disturbances to it, without using animals.

Which non-animal alternatives did you consider for use in this project?

A number of non-animal alternatives will be used as part of the outlined studies, including:

- Cultured cell lines engineered to carry a protein of interest, often an ion channel.
- Stem cell-derived cells that have some of the characteristics of human heart muscle cells.
- Computer simulation of the electrical function of the heart that underlies normal heart rhythm (this in collaboration with specialists in biological physics and mathematics).

However, as of yet, such non-animal alternatives do not enable the fundamental questions relating to the cellular and sub-cellular mechanisms underlying heart rhythm to be addressed.

Why were they not suitable?

Cells grown in culture are of limited value in these studies as they rapidly lose the ability to spontaneously generate the electrical signals responsible for heart rhythm and also lack the anatomical and physiological criteria needed to address the fundamental questions relating to the cellular and sub- cellular mechanisms underlying heart rhythm. Data generated from these studies will contribute to the development of computer simulation models however, as of yet, these are not sufficiently advanced to address the fundamental questions that are the focus of the outlined studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of animals is based on previous experience and reasonable expectation of obtaining definitive result. The experiments on this project exclusively use either excised beating hearts (ex vivo) or isolated heart muscle or heart muscle cell preparations (in vitro) and do not involve experimental intervention to the animal (such as drug treatment, recovery surgery). Thus, the project objectives are framed within what would reasonably be expected to give a clear result on an important scientific question and be achievable within the time available. The numbers of animals to be used are those required to meet these objectives. In the case of the numbers of mice to be used, this takes into account the breeding of colonies of genetically altered animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

By definition, the process of good experimental design requires the estimation of the minimum numbers of experiments necessary to achieve the study objectives. In calculating the numbers of animals required, we have drawn upon our previous experience using the in vitro and ex vivo approaches that will be used during these studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

From the previous work, we have gained much experience in the efficient management and breeding of genetically altered strains of mice. The laboratory experiments will be coordinated to ensure that the available tissues from each animal contributes to as many different aspects of the study as possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animals will only be used to provide tissues for studies performed after they have been killed humanely. Suffering will be kept to a minimum and will not exceed that of mild transient pain caused when given an injection or (mice from genetically altered colonies only) collecting a small biopsy sample.

Guidance on the handling of animals and use of anaesthetics are followed to ensure that the techniques used are the most refined possible.

Why can’t you use animals that are less sentient?

The structure and function of heart muscle cells from embryonic or neonatal hearts is immature and unsuitable for these studies as the ultrastructure of mature heart muscle cells plays an important role in the mechanisms underlying normal heart rhythm and in disturbances to heart rhythm. It is therefore necessary to use hearts from adult animals.

Major structural and functional differences exist between the hearts of mammals and lower vertebrates, such as frog’s or fish, including: the number of ventricles, the conduction velocity of the conducting system and the structure and function of the heart muscle cells. Consequently, it is necessary to use mammalian hearts for this project.

Rats and mice are well-established models for the study of heart function and are therefore appropriate for many of the objectives of this project. Mice are also suitable for the study of the effects of genetic alteration on heart function. However, the rabbit and the guinea pig are the more appropriate species for the study of the conduction system and for the study of abnormalities in heart rhythm arising in the ventricles.

Many of the animals used in this study will only undergo tissues harvested under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures on this licence involve no more than mild transient pain associated with an injection or (mice from genetically altered colonies only) the collection of a small biopsy sample. Suffering during these procedures will be minimised by the use of the most refined methods and good animal handling using non-aversive methods. Where animals are injected, new needles will be used on each occasion and needles will be discarded after

use (i.e. needles will be used only once). The welfare costs to the animals will be kept under regular review and any refinements that improve their wellbeing will be implemented.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Local Policy and Guidance on animal use and handling will be adhered to at all times. The injection techniques used will comply with that described in NC3Rs and LAVA guidance documents. Needles will be used only once (no re-use of needles) and non-aversive handling methods will be used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Establishment administration regularly circulates information, both via email and via newsletters, to PPL holders regarding activities and seminars run by the regional NC3Rs representative. In addition, we receive communication directly from NC3Rs and through the national establishment network. This information is then disseminated to members of the group, encouraging participation and with advice on implementing advances where relevant and appropriate.

20. Development of combined treatments for cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, oncolytic virus, immunotherapy, vaccination, drug-resistance

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project is a continuation of our past and current work with the primary aim of finding new treatments for cancer using animal models. Such studies are necessary and important for future testing in cancer patients in clinical trials. Our research is focused on cancers that that are difficult to treat and have poor outcomes such as pancreatic, lung, ovarian, breast, prostate, bladder and brain tumours. Many of these new treatments involve the use of specially modified viruses that kill only cancer cells whilst avoiding normal, healthy human tissue to minimise any unwanted side effects. These viruses will be tested in combination with other anti-tumour drugs, including those that encourage the patient’s own immune system to help further boost cancer cell killing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a major health issue causing much suffering and death worldwide. Despite recent treatment discoveries that improve patients' quality of life, most do not cure cancer. Many cancers become resistant against treatment over time and effectively overcome the initial cancer-killing effects to eventually spread to many sites in the body, at which point the disease becomes even more difficult to control. It is therefore essential to investigate and develop new therapies that tackle these problems.

These will include specifically modified viruses that kill only cancer cells whilst also activating the patient's own defence (immune) system, enabling it to recognise cancer tissue as "foreign material" and destroy it. To identify better treatments, it is necessary to perform animal testing to ensure that the drugs are both safe and effective before evaluation in humans. This project will help us find and develop new ways to prevent and treat cancers, decreasing the suffering in patients.

What outputs do you think you will see at the end of this project?

Short-term: Understanding the mechanisms of action of our novel treatments. We will use our previously developed mouse models of human cancer to determine how our new treatments act on both the mouse and the cancer tissue. These models include mice with and without a functional immune system and mice that have been genetically modified to develop pancreatic tumours. The models are well established and used by research teams world-wide. These models have enabled us to refine treatment schedules and doses of our newly developed cancer-specific viruses. In this project we will use state-of the-art techniques to investigate the role of the cancer-tissue microenvironment (other cells and factors supporting the cancer cells) and the immune system and, how on a molecular level our new treatments can inhibit the growth of cancer tissue. We will also identify biomarkers for both tumour growth and inhibition by our treatments for future clinical applications.

Medium-term: Development of new improved combination-treatments for human cancer. The major goal of our research is to develop novel therapies for cancer, based on a greater understanding of the molecular biology of the disease. The absolute pre-requisite for developing a successful treatment is that it will have anti-tumour activity and safety in live animals (mice). We are continuing our work on understanding how our new biological treatments (viruses) for cancer can improve the effects of currently used cancer drugs. Our findings from the previous project demonstrate that combining our viruses with chemotherapy and/or immune therapies results in greater efficacy than either treatment alone. However, we still need to investigate and determine the optimum combinations, the dosing schedules and the mechanisms underlying the improved effects to create robust therapies with clinical potential. During the past 5 years we have made excellent progress in identifying specific modifications of the viruses and the specific immune-factors that are essential for killing cancer tissues. In this project we will continue to explore the underlying mechanisms to enable future applications in patients.

Long-term: Understanding of the role of the immune system in the activity of our new anti-cancer treatments and long-term protection (vaccine development). In the previous project we demonstrated that oncolytic viruses induce long-term cancer-specific immunity, which is critical for the overall efficacy of the treatment. In this project we aim to gain a good understanding of the factors that are essential for immunological memory to also protect patients from regrowth of cancers after treatment. For example, we will continue to assess the potential of using antigens (whole cancer cells, protein, peptide, DNA or RNA), viruses or immune cells both as treatments for established cancer and for protection for future cancer growth (development of vaccines). Our research is aimed at directing an immune attack on cancer-specific proteins by exploring large scale patient data bases (existing knowledge of cancer specific proteins) for a number of cancers. This will enable us to further optimise our virus-based treatments.

Towards year 3-5 of this work we anticipate to have several publications in press to share our findings with both the scientific and medical communities as well as patient organisations including our Patient and Public Involvement Advisory Group (PPI) at our Institute.

Who or what will benefit from these outputs, and how?

The findings from our studies will guide future research to better target cancers. We intend to publish our findings in professional journals and present all results in seminars for access by other researchers, medical professionals and patient groups, continuously during the 5-year project. We are funded by charities for pancreatic and prostate cancers that both have interactive patient organisations that visit and review our progress. Our Institute also has an active Patient and Public Involvement Advisory Group (PPI) that meets to discuss projects and proposals.

The major benefits of this project are that our findings have the potential to be directly translated into clinical trials for our novel treatments in patients with a wide range of cancers (at the end of the 5-year project).

How will you look to maximise the outputs of this work?

We have ongoing collaborations with both national and international teams focused on cancer research and oncolytic viruses. Through these collaborations we share data including problems, unsuccessful approaches and the development of better in vivo models.

We frequently attend professional meetings to keep up to date and discuss our findings, and we present our work in seminars to other researchers including medical professionals and scientists. We will keep non-experts informed of major findings through online publications and presentations and in meetings with our Charity funders.

Species and numbers of animals expected to be used

- Mice: 6500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice with either normal or defective immune system that are supplied by authorised breeders. Animals with defective immune system will be used for growth of human cancer tissue or cancer cell lines to determine both efficacy and mechanisms of action of our novel therapies. These animals have been established by genetic alterations and do not have harmful clinical effects. The immunocompetent (normal) animals will be used when determining interactions of our novel therapies with the host immune system and whether long-term anti-cancer protection (vaccination) can be achieved. In some studies, we will also use animals that have been genetically modified to develop cancers spontaneously, similar to cancer in patients, such as mice predisposed to pancreatic or prostate cancer. All strains that we use are well established and produce animals without clinical effects. Our studies will only include young adult mice, from 5 weeks of age at the start and most studies ending at 4 months of age. In the long-term protection (vaccination) studies healthy animals may be kept up to 15 months for evaluation without further treatments.

Typically, what will be done to an animal used in your project?

Animals not predisposed to spontaneous tumour development will be inoculated with human cancer tissue, or human and mouse cancer cell lines to establish cancer growth either under the skin or in the relevant organ (for example, the pancreas) or body cavity (for example, peritoneum, bladder). Tumour bearing animals will be treated with anti-cancer agents by direct injection into the tumour or through delivery via oral administration or the blood stream or into the peritoneum. The anti-cancer treatments include our new oncolytic viruses alone or in combination with conventional anticancer drugs (for example, chemodrugs) or immunotherapies (for example, antibodies, cytokines and immune cells). The efficacy and body distribution of our new therapies are evaluated by tumour measurements and imaging of live animals at different time points. These studies are short and will be completed within 1-4 months after the first treatment. We will use imaging whenever possible to monitor treatment success which will reduce the number of animals in each study. During the 5-year period for the project we anticipate the use of less than 6500 mice spread over six protocols.

Some studies require surgical procedures such as injection of cancer cells directly into an organ (for example, the pancreas). For these studies animals will be anesthetised prior to and during surgery and treated with pain relief medication post-surgery. Aseptic

techniques are routinely used at our center and we use approved anaesthetics and pain medication after discussions with our Named Veterinary Surgeon (NVS).

Each animal will be inoculated with cancer cells or tissue once or twice and treatments will not exceed 21 doses in the lifespan of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

From our past experience we predict that the planned studies will cause only mild or moderate discomfort. In the majority of studies, the animals return to normal activity within minutes after treatments such as cell injections or drug administration. When animals have been subjected to surgery the recovery time is usually longer (2-3h after administration of anaesthesia and pain medication). We are and will be monitoring treated animals daily and do not expect any severe side effects. However, if an animal appears to suffer for example, by loss of weight, not eating or drinking, shivering, not grooming, is lethargic, has a hunched posture or display any other abnormal signs, it will be humanely killed using regulated procedures. At the end of each study when all results have been collected, all animals will be killed by humane and regulated procedures and their tissues used for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In our two breeding protocol (up to 2000 animals) we do not expect any clinical effects and from experience these protocols are therefore of mild to moderate severity with the majority of animals experiencing sub-threshold severity in the mild protocol, and the majority of animals experiencing mild severity in the moderate protocol.

All other protocols (up to 5000 animals) have either mild or moderate severity. We do not expect any severe effects in our protocols.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

All the new anti-cancer therapies that we plan to investigate in this project have been, or will have been, extensively tested in the laboratory before we test them in live animals. Our laboratory experiments will identify therapies that are most likely to be successful in patients and will supply us with invaluable information. However, laboratory-based models cannot predict how the new therapies distribute within an organism or how they affect a living animal for example, liver uptake, accumulation in normal tissues rather than tumours and activation of the immune system. These aspects can only be assessed in realistic models of cancer in whole animals.

Which non-animal alternatives did you consider for use in this project?

In order to minimise animal usage, we are utilising highly sophisticated in vitro models, including 3- dimensional organoid cultures, 3-dimensional tumour-stromal cell co-cultures (in contrast to routine growth of cells on plastic in 2-dimensions), culture of primary tumour cells from patients and whole tumour ex vivo cultures, and will continue to do so throughout the lifetime of this project licence. We are also integrating our laboratory research with the extensive bioinformatics platforms available at the Barts Cancer Institute that enables us to refine treatments further before in vivo analysis begins. Only new therapies that demonstrate repeated efficacy in the laboratory using these and other models will be further examined in live animals. We have already minimised the number of animals that will be needed by developing these non-animal models and will continue to improve on ex vivo/laboratory models in parallel with this project.

Why were they not suitable?

Although these laboratory-based techniques are the most sophisticated mimics of the tumour and tumour microenvironment that are available, they fail to model critical aspects of the biology of novel anti-cancer agents. These include bio-distribution, pharmacokinetics, pharmacodynamics and interactions with liver, spleen, kidneys and immune systems. These aspects can only be assessed in realistic models of cancer in whole animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on our previous and ongoing studies. We have extensive experience of studying oncolytic viruses in murine models and over 20 years of collected data on suitable models and number of animals per group to generate reliable data. The studies in this project will utilise these models while advancing our work to improve on both

models and therapies. The work is now at a stage where we will need to focus on the interactions of our novel therapies with the tumour microenvironment and the host immune system. These studies require testing in several immunocompetent mice models (mice with active immune system) necessitating the breeding of transgenic animals (genetically modified mice; estimated to 1500) and immune studies (estimated to 4000). The remaining 1000 animals will be sufficient for testing efficacy and bio-distribution of various combination therapies (including novel therapies and conventional therapies). However, these are the maximum estimated number of animals and in each study an exact calculation will determine the numbers to achieve statistical significance. Whenever possible we use data obtained from previously control-treated animals, for example, when a same agent is tested under the same condition and in the same mouse species, this allows for fewer animals in each study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All studies are reviewed by me as Project Licence holder, including statistical assumptions and calculations. We routinely use data from our past studies to estimate the minimum number of animals required to address the stated primary endpoint of the experiment without having to repeat entire studies. We also refer to other available tools such as the Experimental Design Assistant available from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the 'resource equation' based on the law of diminished return and for larger studies the online power calculation tools. By undertaking studies with adequate statistical power, definitive positive or negative data can be generated from a single experiment, thus avoiding repeated small under-powered experiments that may be futile and wasteful. Whenever possible we use data from control treated animals for example, when the same agent is tested under the same condition and in the same mouse species.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The studies will only include agents that have been pre-selected as potential new therapies using our sophisticated in vitro and ex vivo models of cancer as detailed in the replacement section.

We have incorporated in vivo imaging where possible in the majority of our protocols to enable smaller cohorts of animals to be observed prospectively throughout an experiment rather than relying on killing of separate groups of animals at each time point. This is especially true in models of ovarian and pancreatic cancer, where estimation of tumour burden in the peritoneal cavity was essentially impossible in live animals before bioluminescence and Magnetic Resonance Imaging (MRI). Our imaging team has acquired a small animal MRI unit that we are and will use to determine internal tumour growth. We have developed advanced procedures to image bio-distribution of our new therapies by small animal SPECT/CT that significantly limits the number of mice that are

needed for these studies. The use of imaging in our protocols will avoid the waste of entire cohorts of animals.

By first exploring conditions in small pilot experiments the final conditions can be optimised and also reduce the number of animals subjected to unexpected side-effects. When possible, the data from the same groups of control animals (untreated or mock-treated) will be used in several studies to minimise the number of animals. Tissue is and will be harvested from each study for laboratory investigation to collect all available information from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All experiments will be performed in mice. This is the species on which the greatest amount of information is available. They can be easily genetically manipulated and a vast array of experimental tools (for example, tumour cell lines, antibodies, and genetically altered animals) is available. The models we use in the majority of protocols include growth of cancer cells under the skin of the animal which is minimally invasive and cause limited suffering and distress. When tumours form they are easily visible and can be injected directly with the therapeutics causing only mild to moderate distress. However, we will also use models that better represent human tumour growth. The most simple of these involve injection of tumour cells into the peritoneal cavity; the disseminated growth within the cavity provides an accurate model of human ovarian cancer, with multiple peritoneal and serosal deposits, with formation of ascites. We have also developed orthotopic tumour models, where cells are injected directly into the relevant organ, for example the pancreas. The tumours that develop accurately recreate the complex tumour-stromal interactions seen in the human disease. Although these models require more invasive procedures, the animals recover very rapidly (within hours) and administration of pain relief medication reduces pain and limits distress. Furthermore, results from orthotopic tumour models or genetically altered animals with spontaneous tumour formation will generate more relevant information that in the long-term will reduce both the number of studies and suffering of the animals.

All our studies are classified as mild to moderate.

Why can’t you use animals that are less sentient?

These models are the most realistic available and reflect the most up-to-date information about human cancer biology. More immature life forms are not suitable due to the great differences between humans and for example worms, flies, and fish, and the lack of relevant circulatory and immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In the course of the current project we have significantly refined our procedures with the help of our dedicated Veterinarian, the facility managers and the animal technicians. Further education of animal license holders is an ongoing process with a minimum of one refresher seminar yearly. We now have reinforced daily monitoring of each animal by the responsible person(s) in ongoing studies observing weight and health and making sure that the animals will get used to handling. Surgical procedures and postoperative care including pain management is always discussed with the veterinarian if the study has not previously been performed. We have also moved away from each individual performing advanced procedures such as surgery and have instead dedicated well-trained technical personnel for these procedures. This has greatly reduced the number of animals in each study by decreasing surgical failure and also improved on the generation of reproducible data. Another refinement is the continued and increased use of imaging in our studies that is also performed by dedicated personnel to minimise failure. Using imaging enables us to follow each animal over longer periods of time rather than harvesting tissues from numerous animals at several time points. The use of imaging together with endpoint analysis of tissue enables us to maximise the information obtained from each animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Guidelines for welfare and use of animals in cancer research; Workman P. et al., 2010, British Journal of Cancer 102, 1.555-1557

M.F. Festing, Design and statistical methods in studies using animal models of development, ILAR J, 47 (2006) 5-14. 18

J. Charan, N.D. Kantharia, How to calculate sample size in animal studies?, J Pharmacol Pharmacother, 4 (2013) 303-306.

To plan animal studies: A.J. Smith et al. PREPARE: guidelines for planning animal research and testing. Laboratory Animals (2018), Vol. 52(2) 135–141 and; A.J. Smith et al., The PREPARE Guidelines Checklist Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (https://norecopa.no/media/7893/prepare_checklist_english.pdf)

How to refine the procedures of administration, for example; from The joint working group on refinement, Laboratory Animals 2001.

The directions for reporting of actual severity levels, for example; Smith et al., Laboratory Animals 2018.

The Humane Endpoints, for example; Wallace, ILAR 2000.

All recent advances and practices on NC3Rs website; https://nc3rs.org.uk The ARRIVE guidelines; https://arriveguidelines.org/resources

The Home Office regulations (online material)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My team is on mailing lists from the 'National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs)' and are regularly informed of seminars and workshops that train users in new techniques, and inform on regulations. Our animal facility managers and our dedicated Veterinarian Surgeon regularly update us on new applications and regulations. All researchers that perform animal studies have a personal licence, are continuously trained and supervised until they have demonstrated the requisite competency and are required to attend yearly update seminars delivered by the veterinarian and our skilled animal technician team. I am, together with my senior managers, attending yearly update courses and refresher modules focused on animal welfare in research and improvement of study design. We discuss the new developments with the veterinarian, animal technicians and relevant personnel to explore if and how we can implement new advances.

21. Control of blood flow and cell function in the brain and other organs, in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Capillary pericyte, Dementia, Stroke, COVID-19, Diabetes

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	adult, aged, juvenile, neonate
Hamsters (Syrian) (Mesocricetus auratus)	adult, aged, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how blood flow in the brain and other organs is controlled by different cell types, how blood flow control and tissue cell function goes wrong in disease, and to develop therapeutic approaches to correcting these defects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There is increasing evidence that defects of blood flow control contribute to a wide range of disorders. Heart attack and stroke are major diseases where blood stops being provided to the relevant organ. In addition there is increasing evidence that defects of blood flow control contribute significantly to dementia (such as in Alzheimer's disease) and to diseases of peripheral tissue such as the kidneys.
Furthermore brain blood flow falls in patients with COVID-19 and this may contribute to the neurological problems associated with this disease. All of these diseases have massive socio-economic consequences.

What outputs do you think you will see at the end of this project?

We will obtain new information on a range of mechanisms regulating blood flow in the brain and other organs, how this affects the functions of the cells in those tissues, and in particular how this contributes to causing dementia. This information will be made generally available to other scientists in the form of peer-reviewed publications.

Several of our experiments explicitly address potential therapies to reduce the occurrence of dementia, stroke, COVID-19 and diabetes. Depending on how successful these strategies are, it may be possible to develop drug approaches to treating or preventing some of the deleterious effects of these conditions on the brain and other organs.

Who or what will benefit from these outputs, and how?

In the short-term (2-4 years) we anticipate our research feeding into the research of other scientists in order to gain a better understanding of mechanisms regulating blood flow in the brain and other organs, and a better understanding of the causes of dementia. In the medium-term our research should lead to

pre-clinical trials of therapeutic approaches to reduce dementia and other disorders (4-5 years). In the long-term (>5 years) we hope that the research will have led to successful therapies in the clinic.

How will you look to maximise the outputs of this work?

We aim to publish all the results of our research, including negative results if they are important. We disseminate our work as preprints, as published papers, as talks to scientists and interested members of the lay public, on social media and in advice given to bodies such as the Medical Research Council and Wellcome Trust.

Species and numbers of animals expected to be used

- Mice: 6000
- Rats: 3250
- Hamsters (Syrian) (Mesocricetus auratus): 585

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.
Types of animal

Mice are selected because they allow the use of transgenic methods to mimic disease, to identify certain cell types with fluorescent labels, and to manipulate cell function by changing the expression level or function of proteins.

Rats are used when a larger animal facilitates some experiments, typically involving cannulation or occlusion of blood vessels or laser Doppler measurements or some imaging experiments.

Hamsters are used because, unlike rats and mice, they are suitable for experiments investigating the effects of the virus causing COVID-19, owing to the sequence of their ACE2 (an enzyme expressed in pericytes which is the receptor for COVID-19) being much more similar to the human ACE2 sequence than are the rat and mouse ACE2 sequences.

Choice of life stage

Young animals (in the first 4 weeks of life) may be used because developmental processes are occurring then that we may wish to investigate, such as the formation of synapses between neurons. They also offer technical advantages in terms of the optical clarity of the tissue for imaging, and ease of electrical recording. Adult and aged animals will be used in order to investigate diseases of adulthood, particularly those leading to dementia in old age.

Typically, what will be done to an animal used in your project?

Some transgenic animals (i.e. mice with cells labelled with a fluorescent dye for identification purposes, or with protein level altered to investigate its function) will be bred (including clipping their ears for identification purposes) and then will be humanely killed, in order to study their tissues.

Some animals (which may also be transgenic mice) will be bred, and may have dyes injected to label the blood or cell types or drugs injected to try to prevent diseases developing, and will then be anaesthetised and their brain or other organ will be imaged (e.g. through a thinned skull or a hole in the skull) while under anaesthesia (and they will finally be put to sleep while anaesthetised).

A much smaller proportion of animals will be repeatedly studied - using tests of memory and/or by imaging the brain through a thinning of the skull (or a hole in the skull that is permanently covered with a glass window) - as a pathological condition like Alzheimer's disease develops, and drugs will be used to try to prevent the decrease of brain blood flow and changes in nerve cell properties that occur in this disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Many of the procedures that we will use have no significant effect on the animals' lives. This includes routine breeding of animals, and rearing them to an old age; administering harmless dyes or protective drugs to the animal; and assessing cognitive function.

Some animals will have a window implanted into their skulls or will have their skull thinned for imaging, or else will have viruses injected into their brains to change the manufacture of proteins in cells. This is unlikely itself to significantly affect the animal, but a small proportion of animals may suffer ill effects for a few days on recovering from the surgery involved, such as having infection of the wound which will be treated with antibiotics (and in extreme cases if necessary the animal will be humanely killed).

Some animals will be mice that are genetically manipulated to produce a condition that mimics diabetes or Alzheimer's disease, and they will gradually experience, for diabetes, modifications of control of body sugar and an increase in urination, and for Alzheimer's disease, a decrease of their mental abilities - one of the aims of the project is to find ways to prevent this happening.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice

- Mild 85%,
- Moderate 15%

Rats

- Non-recovery 25%,
- Mild 64%,
- Moderate 11%

Hamsters

- Non-recovery 28%,
- Mild 54%,
- Moderate 18%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For the great majority of experiments, when studying blood flow, or interactions between neurons, glia and the blood vessels, it is essential to use tissue in which all the cell types have their normal spatial relationship. Consequently cultured cells cannot be used. For

example it is not possible to grow cultured blood vessels connected to an operating heart that functions as in the real body.

Which non-animal alternatives did you consider for use in this project?

Our main approach to non-animal alternatives is to try to use live human tissue when possible. We obtain such tissue from surgical operations (e.g. on brain tumours) in which some tissue needs to be removed to access an area of interest (e.g. a tumour or epileptic focus). This overlying tissue would normally be discarded, and we are able to use it for experiments (ethical permission is in place for this).

As an alternative non-animal approach we routinely employ mathematical modelling of cell interactions, which we have made freely available to the scientific community on the worldwide web.

Why were they not suitable?

The main difficulty of the human tissue approach is that the tissue is only available in very limited quantities, and furthermore the source of the tissue does not have the same consistency of properties as animal tissue does (due, e.g. to age variations or prior drug treatment). Nevertheless, significant results can be obtained with this approach, and we will continue to expand this approach in our work.

Mathematical modelling can provide useful insight into complex problems, but in general cannot replace actual experiments on biological tissue if it is necessary to learn new facts about how individual signalling pathways operate. Thus, mathematical modelling is inherently limited in scope.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

By estimating:

(3) the number of separate experiments we need to perform (including for exploratory investigation of a scientific area, for pilot experiments to establish effect size, and for experiments testing specific hypotheses),

(2) the number of animals needed per experiment (given hypothesised effect sizes), and

(3) the number of animals we need to breed to produce these animals (e.g. by crossing different types of transgenic mice),

and then subtracting animals that we can avoid using by having different investigators share tissue from the same animal.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The major step is to take into account the sharing of tissue from a given animal between different researchers. This can reduce the number of animals needed for some experiments by a factor of 2 typically. However, it is often not applicable for experiments imaging animals in vivo because having different researchers work on the same anaesthetised animal would prolong the experiment excessively.

In addition, the use of transgenic mice significantly decreases the number of animals needed, because it allows the labelling of specific cell types. This avoids the need to record from cells before knowing their identity and only identify them later using immunohistochemistry, which itself is often unreliable. It thus avoids the need to redo experiments when the cells turned out to be different from what was believed at the time.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

There is a strong culture of reducing animal use in my laboratory.

The major animal reduction measure is the sharing of tissue from a given animal between different researchers. This can reduce the number of animals needed for some experiments by a factor of 2 or 3. However, it is often not applicable for experiments imaging animals in vivo because having different researchers work on the same anaesthetised animal would prolong the experiment excessively.

We also try to monitor closely our breeding colonies of transgenic animals, in order to keep to an absolute minimum the number bred.

We also carry out some experiments on live human tissue when it is available from surgical operations, and perform some ‘experiments’ by computer modelling.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodent models that allow us to image blood flow in the brain and other organs, to investigate how defects in blood flow control lead to malfunction of the brain and other organs. The methods used include making tissue slices from tissue taken from dead

animals, imaging of the brain and other organs in anaesthetised animals, manipulating cell function using genetic or drug methods, and assessing brain function using memory tests.

The memory test used, the Barnes maze, offers advantages over the commonly used Morris water maze in that it does not involve swimming and the potential confounding factors associated with it.

In order to study processes that lead to pathology in diseases of great social and economic impact - such as Alzheimer's disease and stroke - it is impossible to avoid creating some suffering in the animals used, but we strive to keep this to a minimum by using humane killing and general anaesthesia. Thus, many of our invasive experiments are carried out on animals that are terminally anaesthetised or have already been humanely killed to obtain their tissue. At the end of each experiment, or set of experiments, on an animal it is humanely killed.

Why can't you use animals that are less sentient?

Mice, rats and hamsters will be used for this project as they represent the least sentient species appropriate for this type of work.

In addition, decades of research have resulted in highly advanced and efficient techniques developed for rodents as opposed to other species. This includes excellent stereotaxic maps of the mouse brain, allowing accurate targeting of injections to specific brain regions, and the mouse is also high genetically tractable, allowing transgenic identification of specific cell types crucial to the fulfilment of the project .

Many of our invasive experiments are carried out on animals that are terminally anaesthetised or have already been humanely killed to obtain their tissue.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Appropriate anaesthetic/analgesic regimens will be used to minimise pain. e.g. delivery of pre- operative analgesia, as discussed with the NVS and Animal Facility Staff.

Maximum injection / infusion parameters will be strictly adhered to. In the very unlikely case that this is not possible due to extenuating circumstances, any changes will be discussed with the NVS.

Housing cages will be spacious and enriched with e.g. rodent toys, chewable materials such as wood, running wheels, and shelter, unless these interfere with the experimental design. In addition, where possible animals will be group housed post surgery using strategies devised in collaboration with our local NVS and NACWO.

Our behavioural tests will not use food or water deprivation.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Information from the local NVS and NACWO Resources hosted on the NC3Rs website, in particular:

- ARRIVE guidelines on experimental design and reporting results.

- 'Procedures with Care': 'Aseptic Technique in Rodent Surgery'.
- Rodent housing and husbandry.
- Rat and Mouse Grimace scales.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep in constant contact with the local NVS and NACWO to ensure we are maintaining best practice.

We will liaise with the in-house NC3Rs representative to ensure we are up to date with current best practice.

We will use the resources published on the NC3Rs website to ensure that the group undergoes continuous training and professional development with respect to the 3Rs.

We will follow technological advances in the published scientific literature, allowing more efficient recording and imaging techniques (yielding more data per animal), miniaturising equipment (leading to a refined animal experience) or allowing recording or imaging in more naturalistic settings (for example, via wireless data transmission).

22. Assessment of biological response to novel ocular drug delivery formulations and devices

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Eyes, Drug delivery, Nanomedicine

Animal types	Life stages
Rabbits	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to assess the biological response to novel ocular drug delivery formulations and devices for the treatment of sight-threatening eye diseases. It will assess whether these implanted formulations and devices cause any undesirable response in the eye. It will also assess whether they are able to deliver drugs to the appropriate tissues in the eye at a controlled, predictable concentration at doses that are likely to be clinically effective and over sufficiently long periods of time.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Delivery of drugs by injection direction into the eye is currently the most effective route for treatment for several common, sight-threatening eye diseases, including diabetic retinopathy and age-related macular degeneration. Hundreds of thousands of these injections are done every year in the UK, and patients have them for many years, so numbers are increasing. This creates a significant financial burden to the NHS and impacts negatively on patients, who have to attend clinic appointments and endure an uncomfortable procedure on a regular basis. There are certain patient groups for whom this is even more challenging, particularly the elderly, those in rural areas and those with dementia. In low/middle income countries, where there has been a rapid rise in the prevalence of diabetes, repeated injection is not achievable. Intravitreal injections also have a risk of sight-threatening complications.

The development of drug delivery technology that can reduce the number of injections would have significant clinical, societal and economic benefit. We have drug delivery systems that show promising results in benchtop studies, and now wish to see if these perform safely and can deliver drugs appropriately inside a living eye.

What outputs do you think you will see at the end of this project?

For our silicone oil technology, we will conduct studies to demonstrate that the oils are biocompatible. The outputs will be used by our group and the medical device industry, particularly our collaborators, to develop silicone oil-based medical devices that can deliver drugs. They will also be used to produce scientific publications and inform future research projects.

For the nanomedicine and hydrogel formulations, we will generate data on the biocompatibility and, in some cases, intravitreal release behaviour of various formulations. We will also gain insight into whether the drugs leave the eye and enter the rest of the body; this is an area in which knowledge is lacking. It is likely that these data will be used as part of the case for supporting protection of Intellectual Property.

Who or what will benefit from these outputs, and how?

The outputs from silicone oil studies will be used by our group and the medical device industry to develop silicone oil-based medical devices that can deliver drugs. These will be used as they become available. They will further add to the academic group's position as world-leading in the development of novel tamponades. Subsequent clinical trials would likely be carried out with local clinical partners, providing benefit to the reputation of that establishment, but also to its patients, who would have the opportunity to be the first to receive new treatments.

The outputs from nanomedicine and hydrogel studies will help clarify whether these new technologies are suitable in this application. They will help us to screen out certain formulations and accelerate development of those with the most potential to be developed

into a clinical product. We will also use pharmacokinetic data in development of our in silico models, in order to accelerate development of future formulations, and to reduce animals required for their testing by screening out unsuitable formulations. The outputs will, therefore, be used immediately. Our team includes academic clinicians, who will generate and understand data that will allow them engage directly with patients' concerns over nanomedicines.

New technologies for ocular drug delivery will benefit patients by providing a means of long-acting release formulations that can reduce patient discomfort and inconvenience and bring treatments to new patient groups. At the same time, healthcare systems will gain a cost-effective means of delivering treatment and improving clinical outcomes. Drug delivery systems could also be useful in veterinary applications, as diabetic retinopathy and uveitis are significant causes of sight loss and frequent intravitreal injection causes distress. These benefits are likely to be realised in ten-fifteen years.

How will you look to maximise the outputs of this work?

The UK is already a leader in both nanomedicine and ophthalmic biomaterials and, through publication of the data generated within this project, it is anticipated that we can strengthen this position and develop collaborations with national and international research groups. Protection of intellectual property will be sought where appropriate, to increase the chance of commercial development of the technology and, thus, the likelihood of it reaching patients. Dissemination of materials, techniques and skills will occur as appropriate. The demonstration of proof-of-concept of safe and effective drug delivery platforms could lead to collaboration with other partners with biological assets, potentially current clinical drugs where reduced frequency of intravitreal injection could improve clinical burden and patient welfare

Part of the existing funding for this project comes via a scheme specifically designed to progress technology towards generating clinical impact. As a consequence, there are detailed plans to maximise outputs. Existing collaborations with both a clinical partner and two industrial partners, built in to the funding, will be used to maximise the outputs. One industrial partner has a track record of commercialising silicone oil technologies developed in universities, and this partnership provides a proven route towards achieving healthcare and commercial impacts from the technology. Their close involvement with the formulations/device design will also help de-risk the next stage of investment. The clinical partner will have the opportunity to input into the project, which will lead to more rapid development of effective treatments for his patients and, if the technology progresses to clinical studies, increase institutional prestige. The clinical partner will also facilitate patient involvement will benefit the project team, and connect patients to research. Interaction with patient groups will increase patient knowledge of advances in treatment, and may encourage their engagement in their own treatment and inspire lobbying for the funding of eye research.

Species and numbers of animals expected to be used

- Rats: 60
- Rabbits: 120

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Pilot studies for nanomedicine and some hydrogel formulations will initially be conducted using adult rats. The use of rodents for these studies is well-established in toxicology, and will allow us to follow guidelines for best practice in design and conduct of the studies, and to take advantage of publishes data to use the smallest number of animals possible.

Studies of how the drugs move in the eye will be conducted in adult rabbits. It is well-established that rabbit is clinically predictable animal model for how drugs move in the human vitreous

Vitreous replacement studies will be conducted using healthy adult rabbits. There is no disease model that can be used. Rabbits are considered an appropriate laboratory model for assessment of how well these devices will be tolerated, as their eyeballs, although smaller than humans, and with a relatively large volume being taken up by the lens, are large enough for performing the required surgery without a high complication rate. A healthy, adult rabbit model has been chosen because it is the lowest species with eyes of appropriate size and structure. This will allow implantation using surgical techniques to be applied that are directly transferable to human eyes. These techniques are well-established, and have a low complication rate.

Typically, what will be done to an animal used in your project?

This project will involve implanting, under anaesthesia, and usually by injection, devices and drug formulations into the eye. These will be left in the eye for up to 12 weeks. The eyes will be studied regularly throughout the experimental period using non-invasive imaging and measurement techniques that will allow assessment of the response to the implanted device/formulation, as well as to assess welfare. Standard welfare checks to assess the general health of the animals will be performed throughout. In some experiments, an invasive imaging technique will be used to assess the function of the retina, which will help with the assessment the response to the implanted devices and formulations. For this, animals will be anaesthetised and have small electrodes placed on the eye and head, which will record the response of the eye to flashes of light.

What are the expected impacts and/or adverse effects for the animals during your project?

Experiment	Animal	Likelihood	Impact/ adverse effects	Duration

All	Rat, rabbit	Low	Post-operative infection Animal showing swelling, redness or discharge at the operation site or reluctance to move, vocalisation when handled or posture suggestive of pain.	The animal will be killed if no improvement is seen in the first 24hrs of treatment or if its condition deteriorates before then.
All	Rat, rabbit	High	Short periods of restraint There may be initial agitation, but the methods of restraint are standard to the handling of rabbits and are well-tolerated by the animal	The periods of restraint will be relatively brief (about 10 minutes maximum)
All	Rat, rabbit	High	Vision loss Surgical implantation of the experimental devices and formulations will have an immediate effect on the animals. It is invasive surgery that will result in some vision loss in one eye. Devices and formulations that cause an adverse tissue response may result in increasing loss in vision that may be permanent.	The vision loss may persist for the duration of the experiment (up to 12 weeks)
Drug loaded	Rat, rabbit	Low	Systemic toxicity due to transport of drugs outside of the eye.	The animal will be killed if signs of systemic toxicity formation are observed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that all rats and rabbits will experience moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to assess the biological response of our devices and drug formulations in the target organ. In particular, we will be assessing safety and drug release from our devices over several weeks. Prior to the commencement of any animal studies, we will use a range of non-animal techniques to assess our formulations and devices. In order to assess the biological response fully, and meet our scientific aims, we need to study how our formulations and devices behave in a complete, living eye, and see how that eye responds to our formulations and devices.

Which non-animal alternatives did you consider for use in this project?

We have already established a range of in silico (computer), in vitro (benchtop) and ex vivo (tissue from dead animals) models that will be utilised during this project to optimise formulations and precise delivery method (e.g. length and gauge of syringe) prior to animal work. This includes protocols for studying release of drug from silicone oil in ex vivo porcine eyes obtained from abattoirs. We have also developed in vitro and in silico models that model drug release from silicone oil and the transport of model compounds across permeable barriers in a flow environment to mimic the outer blood-retinal barrier (a key part of the tissue in the eye that affects how much drug stays in the eye). We have established an ex vivo model for testing aggregation of nanomedicine formulations in porcine vitreous pig eyes and the release of model drugs from these aggregates into the vitreous part of the eye. This is important as it is known that nanomedicines move differently in vitreous than standard drug formulations.

Why were they not suitable?

There are limitations to the in silico, in vitro and ex vivo models we will use in earlier parts of the project. In particular, they suffer from the inability to: -simulate tissue sub-compartments to see how the different of the eye responds faithfully simulate human biology and the processes that are involved in the absorption, distribution, metabolism and excretion of drugs faithfully simulate the in vivo immune response. Available models do not include all the cell types or immune molecules that may have an important impact on the response relationships.

As a consequence, the scientific aims may only be fully met by utilising in vivo models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental and statistical design has been conducted following consultation with biostatisticians, and will we consult further with them as required, and as pilot data becomes available. We have used published data from similar studies where possible to help inform our estimations. These have given us an idea of what size of effect we might expect to see from our formulations, as well as the complication rate. Where there is not enough data available, we will conduct a pilot study to produce data to allow us to make better estimations on the number of animals required for a main study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3Rs Experimental Design Assistant (EDA) has been used to plan workflow and will be further utilised once data from preliminary in vitro, ex vivo and pilot studies are available. We will alter the length of studies if later time points do not provide any additional information. This will reduce the number of animals that are required. The use of non-invasive ophthalmic imaging means that the number of animals required overall is reduced, as multiple measurements can be collected from a single animal throughout the study. Furthermore, each animal has an experimental and a control eye, meaning that the number of animals required for controls will be reduced.

Prior to starting work with live animals, we will conduct a series of in vitro studies to screen out unsuitable formulations. These studies will include tests suggested in ISO 10993-5 (Biological evaluation of medical devices — Part 5: Tests for in vitro cytotoxicity) and ISO 16672 (Ophthalmic implants — Ocular endotamponades), which are relevant to the devices and formulations we are designing.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A likely source of variability is differences in surgical procedure. We will use ex vivo tissue, including tissue from abattoirs and from the establishment tissue bank, in order to optimise and standardise surgical technique prior to using live animals. We follow, as closely as possible, techniques that are well-established in human clinical practice.

Where insufficient data is available, particularly for novel formulations, we will use in vitro methods to attempt to define exposure-response relationships, then conduct pilot studies to generate data to allow us to design subsequent, main studies optimally.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use adult rats and rabbits during this project. Rats will be used for preliminary dose studies for novel formulations. Rabbits will be used for the majority of studies. The size of the rabbit eye makes it amenable to the application of specialist surgical tools and techniques used in human ocular surgery, which should minimise the complication rate and increase the chance of the scientific aims of the project being met. It is possible that surgery may result in post-operative infection or significant tissue inflammation; in these cases, animals will be killed for welfare reasons. Small gauge instrumentation will be used where possible, in order to reduce pain and minimise the chance of post-operative infection

The process of implantation may cause partial or complete sight loss in the operated eye. A key part of our study is assessment of tissue response to the implanted device, so keeping the animals with this inevitable sight loss is required. To reduce animal distress, only one eye will be operated on, leaving sight in the remaining eye.

Why can't you use animals that are less sentient?

Less sentient species, such as rodents, have eyes that are too small for accurate surgical implantation of silicone oil tamponades. Furthermore, the highly-specialised surgical instrumentation required cannot be used in small eyes.

For nanomedicine and some hydrogel studies, we will use adult rats for pilot studies. Smaller rodents, such as mice, have eyes that are too small for such studies. Use of rats will help us screen out formulations in pilot studies. Published data on dose scaling between rats and rabbits are available. For longer term studies, rabbits will be used for technical reasons (it is difficult to isolate rat vitreous) and scientific reasons (rodents have been shown to have low predictive value for certain classes of drug, and their eye is too small for a translatable tolerability study, and published data demonstrating that rabbits are a clinically predictable animal model for intravitreal pharmacokinetics for a range of different drugs).

We need to assess the response to our novel formulations over several weeks, so we cannot use animals that have been terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use our effective, established protocols (developed in our current work, which involves similar surgical procedures to those that are required in this project) to minimise welfare costs for the animals. These protocols include pre-, peri- and post-operative screening for general and ophthalmic health, and an effective pain relief procedure. One advantage of the eye is that it is possible to see inside it; we will use real-time imaging of

operated eyes in order to identify problems with the eye that would mean that the scientific objectives of the experiment could not be met, and humanely kill animals if this is the case.

Animals will be closely monitored throughout all the studies and pain relief will be given to animals showing any signs of pain and/or distress. We will seek advice from the Named Veterinary Surgeon (NVS) and Named Animal Care & Welfare Officer (NACWO) where appropriate. Any animals whose symptoms cannot be relieved will be humanely killed using a Schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use NC3Rs best practice guidance where applicable. In particular, we will use:

- the rat and rabbit grimace scales to detect the presence or absence of signs of pain or discomfort.
- LASA/NC3Rs Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals (for guidance - although our studies are not for regulatory purposes, many of the best practice elements will be the same)
- LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery
- LASA Good Practice Guidelines - Collection of Blood Samples

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My team and I will continue to attend local and online NC3Rs events and in-house training to learn about 3Rs best practice and innovations. This also facilitates access to the NC3Rs Regional Programme Manager. I will also maintain my subscription to the NC3Rs e-newsletter and Tox News. We will also continue to attend specialist ophthalmic research conferences, in order to stay informed about field-specific advances (e.g. imaging techniques) that could have 3Rs benefits for this project. We will discuss these advances with the local facilities team in order to ensure the feasibility and benefit of any proposed changes, as well as maintaining compliance with licence conditions.

23. Provision of services to investigate novel health technologies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

translational, biocompatibility, safety, surgery

Animal types	Life stages
Rats	juvenile, adult
Mice	juvenile, adult
Rabbits	juvenile, adult
Animal types	**Life stages**
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to provide research services to enable the assessment of novel health technologies. These include:

safety and biocompatibility assessments of novel materials,

safety or efficacy assessments of substances delivered to the eye,

devices or substances to treat conditions related to gastrointestinal surgery, bowel anastomosis and chemotherapy delivery.

devices to treat conditions related to the eye

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The models and protocols described in this project are intended for research into surgical and interventional sciences, and will be applicable across a range of clinical conditions which require surgery.

What outputs do you think you will see at the end of this project?

Outputs of research conducted under this licence may include: publications, patents, product development, product registration, commencement of clinical trials, new products.

Who or what will benefit from these outputs, and how?

In the long-term this research will benefit patients with various diseases or conditions: the impact of this work is applicable in multiple treatment fields.

For research Sponsors, data can assist with decision making, for instance elimination of unsuitable candidates and selection of more suitable candidates, prior to further costly development, and further animal research.

Research models using smaller mammals are useful to refine and develop techniques and can generate data upon which further investigative studies can be based, either in clinical trials or in vivo trials. Research models using larger animals are useful to simulate, refine and develop surgical techniques and strategies.

How will you look to maximise the outputs of this work?

Whilst publication of results would not be discouraged, it is expected that in most cases publication or dissemination will not be possible as the research will be conducted as a commercial service to companies.

Species and numbers of animals expected to be used

- Mice: 250
- Rats: 250
- Rabbits: 450
- Pigs: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages

Biocompatibility of materials (Rats & mice)

Rats and mice are used as models for assessing material biocompatibility as materials can be placed sub-cutaneously, relatively simply and safely in a short surgery time (compared to larger animals).

Their smaller size enables the inclusion of larger group sizes or collecting more datapoints more easily in terms of handling and husbandry, improving data reliability, as well as enabling multiple tissues to be assessed post-mortem more easily compared to larger animals. This is an efficient way to investigate or compare new treatments or multiple candidate materials quite quickly. Juvenile or adult animals would be used depending on the Sponsor's requirements, e.g. body weight, growth rate, size of implant/material to be placed, or the intended life span of the implant/material or length of study.

Ocular dosing and ocular tissue regeneration (rabbits)

Rabbits are frequently used for ophthalmic research as the size of the eye is more comparable to humans compared to smaller animals (e.g. mice or rats) and the stem cell regeneration of the epithelium is more similar to humans compared to mice. Substances can be relatively easily delivered into the rabbit eye, compared to smaller or larger animals.

Bowel anastomosis and drug delivery

Larger animals (in this case, pigs) are useful to evaluate products in the final stages of pre-clinical development, or devices which have been scaled up to a representative human size, or procedures intended for human surgery. Larger animals are often used for testing medical devices due to their physiological similarities to humans: devices and implants of a similar size to those which would be used in humans can be applied; in comparison to smaller mammals (mice and rats) there is a larger blood volume and the response to treatments can be evaluated over a longer period of time. In particular, gastrointestinal anatomy is more comparable to humans, compared to sheep for example, making pigs a useful and clinically relevant model for gastrointestinal and abdominal procedures.

Typically, what will be done to an animal used in your project?

Biocompatibility of materials (Rats & mice)

Most animals will undergo surgery, under general anaesthesia, to place up to 4 samples of materials under their skin. Some animals may not receive the implant or may receive an alternative or control material. Depending on the nature of the implant, it may be injected subcutaneously rather than surgically implanted (e.g. pastes or gels): this would not necessarily need to be performed under anaesthesia.

Ocular dosing and ocular tissue regeneration (rabbits) Ocular injury and tasorrhaphy

For most animals, an injury will be created on the epithelium of one eye, either by physically scraping or cutting the epithelium, or by applying a chemical to it (e.g. ethanol). This would always be performed under general anaesthesia. A loose, temporary suture (tasorrhaphy) would be placed to keep the eye closed for some or all of the follow-up period, but would allow observations to be made during this time.

Application or delivery of substances

Substances or treatments may be applied to the eye. These could be as drops or gels, as physical devices such as a contact lens (which may contain test substances). The substance could be delivered directly to the ocular surface with the animal restrained, or it may need to be injected into the eye, in which case the animal would be anaesthetised. The substances may be administered alone or in combination, at multiple times during the study, or may be slow-release substances/devices, releasing the test material over a prolonged period of time. The substances could be applied for experimental purposes (e.g. new treatments) or for monitoring or imaging purposes (e.g. dyes to view different parts of the eye).

Bowel anastomosis and chemotherapy delivery (pigs)

Bowel anastomosis and device placement

The bowel will be cut in up to 4 places and put back together (using stitches, or staples or glue etc). This would always be performed under general anaesthesia. Devices for drug delivery may also be applied to the bowel or surrounding tissue: e.g. sponges, patches etc. These would need to be attached by a suture, clip, glue or another type of fixing.

Application or delivery of substances

Substances or treatments may be delivered to the bowel or surrounding tissue either during surgery or after surgery via an extension tube. Devices applied during surgery may already contain the substances to be delivered over time. The substances may be administered alone or in combination, at multiple times during the study, or may be slow-release substances/devices, releasing the test material over a prolonged period of time. The substances could be applied for experimental purposes (e.g. new treatments) or for monitoring or imaging purposes (e.g. dyes to view blood flow or bowel obstruction, or labelled cells to assess tissue healing).

Treatment of eye conditions (rabbits)

Device placement

Most animals will undergo surgery, under general anaesthesia, to place a device into the eye.

Application or delivery of substances

Substances or treatments may be applied to the eye. These could be as drops or gels. The substance could be delivered directly to the ocular surface with the animal restrained, or it may need to be injected into the eye, in which case the animal would be anaesthetised. The substances may be administered alone or in combination, at multiple times during the study. Some substances may also be applied for monitoring or imaging purposes (e.g. dyes to view different parts of the eye)

Blood sampling (all animals)

If repeat blood sampling is required for the experiment, up to two (usually one) indwelling catheters may be inserted and tunnelled through the skin. This would be done under general anaesthesia.

Alternatively, if blood samples are only required occasionally, they may be taken directly from a blood vessel while the animal is restrained. It may be necessary and less stressful to briefly anaesthetise larger animals to take occasional blood samples.

Control groups

All steps are optional within each protocol: this is to allow the inclusion of control groups where they are required, such as administering substances without creating an initial injury. "No treatment" control groups are very rarely used for biocompatibility studies and the inclusion of these groups in a study would need to be approved by the Institute's ethical review body (AWERB).

End of experiments

Animals will be killed at the end of the experiments. This would usually be by an overdose of anaesthetic, but occasionally we would place the animals under general anaesthesia and retrieve blood and/or organs before killing it.

What are the expected impacts and/or adverse effects for the animals during your project?

Surgery & injuries (general, e.g. placing indwelling catheters, creating eye injury)

There is likely to be pain and some discomfort, and possibly localised inflammation, originating from any injuries created, the surgical incisions and extension tube for blood sampling, if placed. This would be managed with pain relief and dressings.

Wound opening (all protocols, all animals)

Occasionally, surgical wounds may open during the recovery period. This could happen if the animal interferes with the wound (scratching, overgrooming) or if there is an infection. In the cases, where there is no infection (or minor infection) the wound could be re-closed (usually under anaesthesia).

Infection (all protocols, all animals)

Infection is a possible adverse effect for any surgical procedure. This would be managed with antibiotics and dressings.

Creating eye injury (rabbits)

There is likely to be pain, inflammation and discomfort originating from eye injuries and associated suture.

Collars (mice, rats, rabbits)

To prevent animals interfering with wound sites or dressings, collars can be used. However, continuous use of a collar will interfere with the animals natural behaviour, e.g. unable to groom properly, unable to express coprophagy behaviour, leading to digestion problems). The impacts of continuous use of collars should be weighed against the benefit of preventing the animal interfering with the wound and hurting themselves. This would be assessed on a study-by-study basis. Continuous use of a collar can also rub against the skin, sometimes causing sores, pain and discomfort - this is checked regularly and creams or ointments can be applied, where this does not interfere with the study in progress.

Blood sampling

The animals may be averse to repeat blood sampling procedures which we will attempt to alleviate with habituation, training and enrichment during the acclimatisation period. The animal would need to be restrained whenever a blood sample was to be withdrawn. When taken from an indwelling catheter, the time of sampling should be shorter, with relatively less discomfort, than if the sample was to be taken directly from a vessel. In both cases, the impact would be mild and short-term

Application of substances

Occasionally there may be swelling or discomfort from implants placed under the skin. Following e.g. delivery of substances to the eye, or intraocular device placement, there may be pain, discomfort, irritation or inflammation. There is also the possibility of materials or substances causing some toxic effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: 50% mild; 50% moderate

Rats: 50% mild; 50% moderate

Rabbits: 100% moderate

Pigs: 100% moderate

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use anials to achieve the aim of your project?

The models and procedures described in this project licence are intended for research of new healthcare approaches, treatments or devices, with a particular focus on injuries, trauma, or conditions requiring surgery. In many cases, treatment of a condition requires multiple approaches including surgery and drug therapy. The aim of this licence is to provide research as a service which reflects these multiple approaches and adequately tests new technology for healthcare. The range of potential fields that could benefit from this research covers the majority of medicine. It is expected that new technology is tested first in computer models or laboratory settings, but before technologies can be tested in, or applied to, humans, there must be some evaluation of the potential safety and benefit of doing so. To do this adequately usually requires evaluation in a living model - this includes multiple processes occurring simultaneously including breathing, blood circulation, the nervous system, hormonal effects, metabolism, excretion, growth, interactions with surrounding tissue, healing over prolonged periods of time. The safety and effectiveness of a technology would be affected by all these processes, and it is almost impossible to adequately recreate them in a computer or laboratory test.

Which non-animal alternatives did you consider for use in this project?

Where possible we make use of human or animal cadavers to do the initial stages of research, or basic research.

We also expect the research Sponsors to have completed intitial stages of research in non-animal models before commencing animal research.

Why were they not suitable?

Living processes (breathing, circulation, bleeding, metabolism, growth etc) are not present in cadavers. Non-animal computer or laboratory models cannot adequately present the multiple physiological processes involved in recovery from injury, trauma, surgery or disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated animal numbers are based on examples of likely study designs and timespans, for instance:

one study a year using 5 groups of 10 animals per group (<250 total) 6 studies a year using 8 animals (250 total)

2 studies a year using up to 24 animals of each sex (<250 total) 5 studies a year using 6 large animals (<150 total)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Pilot studies with 1-2 animals can be conducted, for example for significant changes to products or procedures, before commencing larger studies - where possible, pilot study data will be included in the main study.

Where possible, multiple materials could be placed for biocompatibility studies.

Where possible, control materials or devices as well as test items can be placed in the same animal which could eliminate the need for a separate control group.

Wherever possible we make use of non-invasive or non-terminal endpoints such as non-invasive imaging or repeat blood sampling, to eliminate the need for additional study groups for different timepoints.

We frequently recommend cadaver studies to Sponsors, and use cadavers for model development and training opportunities.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies with 1-2 animals can be conducted, for example for significant changes to products or procedures, before commencing larger studies - where possible, pilot study data will be included in the main study.

We routinely harvest tissues and organs from experimental animals for research and training purposes.

We work with Sponsors to ensure that the number of animals is enough to generate good quality data and reliable statistics.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Protocol 1: Biocompatibility
Mice, rats
Surgical placement of implants placed under the skin
Surgical placement of a catheter for blood sampling, and tunneled under and then through the skin
The surgery is always performed under general anaesthesia with pain relief alongside. The skin thickness is thinner in rodents compared to larger animals and surgical placement of materials subcutaneously is relatively simple in rodents and the animals recover relatively quickly (ie. usually within minutes following inhalational anaesthesia, compared to hours for larger animals). The surgical site is on the animal's back so the risk of interference and subsequent wound dehiscence is low.

Catheters would be placed if blood samples were required frequently: this would cause less distress than repeated handling, restraint or anaesthesia to withdraw a blood sample. Medicine to relieve pain would always be administered for a few days after the surgery, and longer if needed.

Protocol 2: Ocular injury and delivery of substances

Rabbits
Surgical placement of a catheter for blood sampling, and tunneled under and then through the skin
Creation of an injury on the eye, with a stitch to close the eyelid loosely
Delivery of substances or devices (e.g. contact lens) to the eye
Delivery of substances to look at the eye (e.g. dye)

The surgery is always performed under general anaesthesia with pain relief alongside. Catheters would be placed if blood samples were required frequently: this would cause less distress than repeated handling, restraint or anaesthesia to withdraw a blood sample.

Medicine to relieve pain would always be administered for a few days after the surgery, and longer if needed. Antibiotics can be administered directly to the eye if they were needed.

Only one eye would be damaged in each animal, and applying ethanol to the cornea in a well helps to control the injury size. The chemical injury is less severe than cutting. The animals are all acclimatised to the staff for handling, which makes the observations and administration of substances easier and less stressful after the surgery. The eyelid of the injured eye would be loosely sutured so that it could be opened briefly to administer substances as drops directly to the eye - this would not usually require anaesthesia.

However, for some observations and measurements, it could be necessary to anaesthetise the animal to administer dyes and shine different coloured or fluorescent lights onto the eye. Doing this under anaesthesia would cause less distress than if the animal was awake and restrained.

Protocol 3: Bowel anastomosis and drug delivery

Pigs
Surgical placement of a catheter for blood sampling, and tunneled under and then through the skin
Injuring the bowel, including completely transecting the bowel and re-connecting it ("anastomosis") in up to 3 places.
Placing a device onto the bowel or other associated tissues (for example the stomach, pancreas, abdominal wall).
Tunneling an extension line through the skin connecting to the device, to allow delivery of substances directly.
Delivering substances, such as chemotherapy, to the injured tissue, or to a device.
Looking at the injured tissue or body using CT or ultrasound, and delivering substances to assist with imaging, such as dyes or fluorescently labelled cells.

The surgery is always performed under general anaesthesia with pain relief alongside. The surgery would be performed by a competent surgeon, which could be a practicing medical surgeon or a competent and appropriately trained research scientist, veterinary surgeon or research surgeon.

Catheters would be placed if blood samples were required frequently: this would cause less distress than repeated handling, restraint or anaesthesia to withdraw a blood sample. Similarly, a tube for infusion can be connected to an internal device to avoid having to anaesthetise or restrain the animal to administer a substance. Medicine to relieve pain would always be administered for a few days after the surgery, and longer if needed. Antibiotics would also be administered if needed. The animals would be monitored closely after the surgery with particular emphasis on any signs of bowel problems or infection. Before and after the surgery, the animals could be provided with a different diet to soften the stools and make gut transit easier. They can also receive flavoured rehydration treatments to drink (which pigs quite enjoy).

Protocol 4: Treatment of eye conditions

- Rabbits
- Surgical placement of a catheter for blood sampling, and tunnelled under and then through the skin
- Surgical placement of devices (e.g. stent) into the eye
- Delivery of substances to look at the eye (e.g. dye)

The surgery is always performed under general anaesthesia with pain relief alongside. Catheters would be placed if blood samples were required frequently: this would cause less distress than repeated handling, restraint or anaesthesia to withdraw a blood sample. Medicine to relieve pain would always be administered for a few days after the surgery, and longer if needed. Antibiotics can be administered directly to the eye if they were required.

Only one eye would have a device implanted in each animal. All animals may experience some post-operative pain or discomfort because of the surgery. The animals are all acclimatised to the staff for handling, which makes the observations and administration of substances, as drops, easier and less stressful after the surgery - this would not usually require anaesthesia. However, for some observations and measurements, it could be necessary to anaesthetise the animal to administer dyes and shine different coloured or fluorescent lights onto the eye. Doing this under anaesthesia would cause less distress than if the animal was awake and restrained

Why can't you use animals that are less sentient?

The choice of animal is based on using the least sentient animal which is most clinically relevant and representative of a human and physiological processes, or most appropriate to represent the intended clinical setting.

Mice and rats are well characterised models for biocompatibility studies and are less sentient than larger animals (e.g. rabbits). Rats are usually preferred for this model over mice due to their larger body size, organ size and blood volume, enabling easier placement of materials and placement of multiple materials where appropriate.

Rabbits are frequently used for research into conditions affecting the eye as the size of the eye is more comparable to humans compared to smaller animals (e.g. mice or rats). Where recovery from injury and repair is to be investigated, the regeneration of the rabbit eye tissues is well characterised and is more similar to humans compared to mice.

Some translational studies require appropriately sized animals which are comparable to humans in order to be clinically relevant. The body size, blood volume and growth rates of larger animals are useful for these types of studies, and the ratio of body weight:blood volume is closer to humans than smaller mammals. The bowel of the pig is deemed more representative of the human than the sheep. Also, new procedures, instrumentation and devices may be designed for humans and a representatively sized animal will therefore have to be used.

We frequently recommend cadaver studies to Sponsors where this would meet the study aims, however studies including a recovery period are necessary to evaluate physiological, biochemical and histological responses to treatments. Larger animals are more appropriate for evaluating techniques and devices over longer treatment or recovery periods (weeks-months) compared to smaller mammals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We routinely provide post-operative pain relief and antibiotics, and are prepared to extend the provision of these where necessary. All animals are closely monitored immediately after surgery, and then observed closely in the days and weeks after surgery, for instance their mood, grooming, movement, weight, eating and drinking, how the wounds look.

For recovery studies, all the animals arrive to the facility at least two weeks before studies start (for non- recovery studies, this can be one week, allowing time for physiology to return to normal levels and for any stress associated with the animal's arrival to diminish). During this time staff can start to train the animals if this will be needed for the experiment, for instance to be restrained (small animals) or stand still (pigs) for blood sampling.

Smaller animals (mice, rats and rabbits) will also be handled frequently before the study to get them used to staff and being handled.

All animals are provided enrichment whilst in the facility, such as chew sticks or treats and raised tunnels for rats; raised steps, a hiding place and dietary enrichment for rabbits; chains, balls and bars for pigs to chew.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our facility is GLP-certified and experiments can be performed to GLP-certified standards where required; non-GLP studies are performed to the same standard operating procedures.

The PREPARE guidelines cover many topics that are usually discussed between the research team and Sponsors whilst developing protocols, and an adaptive approach to these will be useful for planning specific studies and continuing the project.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our research staff actively seek opportunities for training and continuing professional development. We circulate the NC3Rs newsletters and take part in IAT and LASA activities. We communicate openly about animal welfare and suggestions and ideas for improvements are welcomed from all staff members.

24. Induction of anti-tumour immunity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunotherapy, White Cells, Carcinogen, Blood Vessels

Animal Types	Life Stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to improve our understanding of the relationship between the immune system and cancer and to use this information to design new immunotherapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To enable the design of new immunotherapeutic approaches to be tested in patients with cancer.

What outputs do you think you will see at the end of this project?

The design of new immunotherapeutic approaches to be tested in patients with cancer.

Who or what will benefit from these outputs, and how?

The main output of the study described herein is a better understanding of the reciprocal relationship between tumour blood vessels and anti-tumour immune responses. The findings will be shared as quickly as possible with the scientific community via open access publications and through talks at conferences and seminars. As mentioned throughout this application, there is a great deal of interest in developing blood vessel targeting agents for cancer immunotherapy. The findings of this study will be directly relevant to these efforts and as such, will be used to inform the design of new early phase studies in patients with cancer.

As well as the expert audience, we will also communicate our new findings to patients and the public. This has previously been done through lab visits for charity supporters, patients and their families, hosting coffee-shop evenings and speaking at public events. We also interact with schools and are specifically involved in projects aimed at helping young people understand the complex relationship between the immune system and cancer.

How will you look to maximise the outputs of this work?

Knowledge gained through work conducted on this licence will be shared with:

Cancer Immunology Researchers: Despite the success of novel cancer immunotherapies, the fact remains that for most cancers and the majority of patients, these therapies do not work. Currently, there is a large emphasis of combination therapies whereby a dual approach of e.g. priming the immune system with radiotherapy, chemotherapy, administration of oncolytic viruses etc is followed by or used concurrently with inhibitors of immune checkpoints. This project will examine the impact of combining treatment targeting blood vessels and immune cells thereby contributing knowledge to the design of novel immunotherapies.

Clinical Academics: This research will identify novel immune intervention which could be tested in early phase clinical studies.

Immunologists: Whilst the focus of this study is entirely on cancer, it is clear that the knowledge gained will also contribute to our understanding of immune responses to infection and especially of immune responses generated in the context of autoimmune disease. Thus, the findings of this study will be of interest to the wider immunology community, interested in understanding how to control of infection and/ or exacerbating or limiting autoimmune disease.

Collaborators: This study will extend our ongoing collaborations with multiple groups around the world. Our key contribution will be in imaging and we will aim to share our

methodological expertise with others. A large part of the process of collaboration is discussion of unsuccessful approaches and how this can be tackled. Thus, the progress we make will accelerate research in our group and beyond.

Species and numbers of animals expected to be used

- Mice: 15,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In this study we will use adult mice in which tumours will be induced through injection of a carcinogen or through injection of cancer cells. One purpose of the work is to understand how the mouse immune system responds to the growing cancer. The second purpose is to test new therapies based on manipulation of the immune system. The mouse provides an excellent model in which to study the relationship between the immune system and tumour growth, since mice are well characterised immunologically and several key features of the mouse immune system resemble those of humans.

Moreover, many genetically modified mouse strains are available which will enable us to examine which components of the immune system help control cancer growth. Use of the mouse models described will also enable us to assess and visualise cancer cell and immune cell movement in the body. This is important as it enables us to work out how cancer cells avoid the immune system and how we can prevent this from happening. Currently there are no lab-based techniques which can recapitulate these processes.

Typically, what will be done to an animal used in your project?

Animals used in experimental procedures will be handled frequently (approximately three times per week). During these times animals will be injected and / or monitored for tumour burden (typically takes less than 30 seconds). In order to mimic the situation for patients with cancer, it will be necessary in some cases, to surgically remove tumours before novel therapies are administered. For this purpose, animals are anaesthetised for around 10 minutes. As well as for surgery, some animals will be anaesthetised in order that their immune cells and / or cancer cells can be visualised using imaging techniques. Typical imaging experiments require that animals are anaesthetised for around 1 hour.

What are the expected impacts and/or adverse effects for the animals during your project?

Many of the mice in this study will have a tumour generated either through injection of carcinogen or through injection of cancer cells. Both methods will result in development of the tumour at the site of injection. In the case of the carcinogen, this is on the hind leg

whereas cancer cells are implanted either under the skin or in the colon. In all cases, tumours are readily visualised either by eye or using an endoscope (colon tumours). Mice will be monitored for tumour burden frequently and tumours will be scored for size, position and, in the case of tumours implanted under the skin, redness and/or irritation.Through good handling techniques, distress caused to the animal from being restrained will be minimised in terms of time and discomfort. Should tumours limit mobility, appear inflamed or reach a maximum permissible size, animals will be killed using an appropriate humane method. In the case where surgery is performed, appropriate analgesics will be administered to control pain. Score sheets are used in all experiments to record signs of distress and weight loss. Appropriate remedial action will be taken immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on working with mouse models of tumour growth since 1999, it is expected that for the majority of animals used in procedures in this project, the severity level will be "Moderate".

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Interactions between the immune system and developing tumours depend on the integration of signals from a number of immune cell types and immune mediators. With this in mind, an animal model is necessary as there is currently no way to recreate these in vivo conditions in an in vitro setting.

Which non-animal alternatives did you consider for use in this project?

Some non-animal alternatives are used in this project. We are currently using organoid cultures in our laboratory which grow in 3-dimensions and which resemble the complex cellular heterogeneity of tumours growing in animals. These are useful for examining the direct effects of drugs on cancers and to examine how immune cells interact with cancer cells. However, their use remains limited by a lack of blood supply to the organoids which limits our ability to study cancer and immune cell movement.

Why were they not suitable?

An animal model is necessary as there is currently no way to recreate these in vivo conditions in an in vitro setting.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental designs to be utilised are simple and have been used, with slight variations, in our laboratory for a number of years. For most experiments, little optimisation of regimes required, keeping the number of animals required as low as possible. As the aim of this research is to identify immunological effects that are both reproducible and unambiguous, most experiments will include both positive and negative control groups. This is important as it enables us to ensure that a given experiment is working (positive control group) and that any effects observed are due to the intervention being tested (negative control group). In the case where a pilot experiment is conducted e.g. to determine the feasibility of an experimental technique or to set imaging parameters, no control group will be required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All PhD students and post-docs in the lab will have performed a course on use of statistics in biology and will be well versed in use of "power calculations". This means that they will be able to use "power calculations" to work out what the minimum number of animals is needed in order for a given experiments to produce a meaningful (statistically significant) result. This is important, not just for making sure that no more animals than necessary are used, but also because using too few mice means that it might not be possible to draw a conclusion from the experiment.

Members of the lab also use a set of guidelines called "ARRIVE" guidelines found on https://arriveguidelines.org/. These are an extremely useful set of recommendations which are designed to help researchers ensure that they perform their animal work in the most robust way possible. The purpose is to enhance the quality and reliability of the findings of individual labs in order that their work can be more easily reproduced by others. These principles form the cornerstone of animal work performed in our lab.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Several measures are taken to optimise the number of animals used:

1. This project will make use of a number of different imaging modalities for measuring tumours which are not visible to the naked eye. This will enable us to carry out longitudinal analyses of tumour growth in individual mice thereby significantly reducing the numbers needed to assess the impact of a given immunotherapy on tumour growth over time.

2. Where possible we will try and use the same control group for multiple experiments – this requires coordinating the activities of different members of the lab in order that experiments can be synchronised. Regular small-group lab meetings are held where researchers working with mouse models present their experimental plans for the month ahead. This information sharing enables the design of experiments where control mouse groups can be applied to different (but obviously related) experimental questions. This is a very powerful way of reducing mouse numbers and increasing the quality of the experiment beyond what one individual researcher can achieve. This approach also facilitates greater oversight of the animals involved in the study with shared responsibility amongst researchers.

3. Randomisation is also used to ensure validity of results. Due to the nature of the tumour models that are used, whereby tumours arise at different times in individual animals, experiments are often performed in sub-groups (enabling randomisation within blocks). This approach builds additional robustness to the experimental findings as any conclusions drawn come from findings which have withstood the natural variation occurring within experimental units.

4. Variation amongst mice / experiments is kept to a minimum by conducting procedures at a similar time of day, using uniform and well validated techniques and equipment. In all cases individual mouse variation e.g. body weight, tumour size etc, are taken into account.

5. Where possible, experiments are performed in a blinded fashion. Animal samples taken to the lab will be coded and key measurements conducted by a researcher who is blinded to the experimental code e.g. measurements of immune cells in tumours. Tumour volumes are measured by technicians who are not aware of what (or whether) mice received interventions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse provides an excellent model in which to study the immune system. Mice are well characterised immunologically, and their immune systems closely resemble those of humans.

Tumours will be induced either through injection of a carcinogen in the hind leg, tumour cell lines in the skin or in the colon using an endoscope. Both procedures involve minimal restraint and little discomfort to the animal. Tumours are monitored frequently either by imaging or palpation allowing us to kill animals by Schedule 1 methods before tumours cause pain / discomfort.

Why can't you use animals that are less sentient?

This work evaluates the dynamic interactions between blood vessels, tumours and the immune system. These interactions, which encompass complex cellular heterogeneity and which require the need to analyse cell behaviour in their natural environment must be performed in an adult animal with similar biological systems to those in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All new techniques (or combination of techniques) are subjected to scrutiny with respect to harms to the individual animal. Score sheets are updated to reflect any relevant observations relating to animal welfare. We also work closely with the animal technicians and NACWO to ensure that all parties are aware of potential adverse effects and how to respond accordingly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE Guidelines are the most useful for this purpose.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We stay informed about advances in the 3Rs (reduction, refinement and replacement) through published literature. An example of a transformative step is the development of cancer "organoid" technology. Organoids are three dimensional structures which can be grown from both mouse and human cancers in the laboratory. They retain many of the features of cancers growing in the body and can therefore sometimes be used for testing e.g. new drugs in the lab without the need for an animal model. For this reason, we are currently developing a bank of human breast cancer and colorectal cancer organoids which will allow us to perform at least some experiments to examine the interaction between immune cells and cancer. Students and post-docs in the lab also attend workshops which are run by the "National Centre for Replacement, Reduction and Refinement of Animals in Research" - this provides the lab with the opportunity to learn about the latest innovations (such as use of organoids as described above) which help

reduce the need for animal models or which improve experimental designs such that fewer animals are needed.

Refinements in imaging procedures have been continuously adopted during the course of our studies. This is important because our findings are otherwise limited to static snapshots of how cells of the immune system interact with cancer cells. Imaging techniques enable us to see how cells move and interact with each other over time. This means that substantially more information can be gained from individual animals. These techniques are usually non-invasive thereby offering a significant refinement to traditional methods.

25. Immune regulation at mucosal and barrier surfaces

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Microbiome, Mucosal surfaces, Inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to investigate and understand the interplay between the host immune system and colonising microbes, and how breakdown of interactions between them can lead to the onset of inflammatory diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding fundamental cellular and molecular pathways underpinning inflammatory diseases at barrier surfaces has already led to new treatments effective in the clinic, many

of which target common pathways to inflammation at several sites, including the intestine, skin and joints. However many patients show little response to current treatments, or response declines over time, meaning that additional treatments are required to suppress pathological immune responses or to induce protective immunity against intestinal pathogens. These studies may aid the development of new antimicrobial approaches and vaccines for treatment of infectious disease as well as identification of new therapies for a number of chronic inflammatory disorders in addition to IBD, psoriasis and arthritis, including type 1 diabetes and multiple sclerosis. Our work is relevant not only to human health as chronic intestinal inflammation is also a problem in the livestock industry, and IBD affects domestic animals including cats and dogs.

What outputs do you think you will see at the end of this project?

The overall aim of our project is to understand how microbes and the immune system interact in health and what goes wrong in disease. We are particularly interested in inflammatory diseases such as inflammatory bowel disease, arthritis and psoriasis, in addition to colorectal cancer, which are increasing in prevalence. Our works aims to understand how these diseases arise and how to better treat them. Outputs may include the identification of both common disease pathways and novel treatment targets. This information will be used to design interventions to promote health, which may be translated to human disease through collaborations with industry partners. A greater understanding of the role of intestinal microbes in disease onset, promotion and protection may also lead to more targeted interventions and design of synthetic microbes as an avenue for treatment. All of this work will be published in peer-reviewed publications, and disseminated to the broader scientific community through conferences.

Who or what will benefit from these outputs, and how?

Inflammatory bowel disease is a chronic debilitating inflammatory disease of the gastrointestinal tract that affects around 0.2% of western populations. Disease affects young people in their prime and current therapies do not cure disease and can lead to systemic immune suppression, leaving patients susceptible to infection over decades. Five years after diagnosis, it is estimated that between 15-20% of patients are disabled to some degree by their disease. Our programme of work which involves fundamental basic science is to identify key immune and tissue pathways that control the host environmental interface at barrier surfaces such as the skin and intestine, and non-barrier areas such as the joints in health and how these change during disease. Based on the success of our previous programmes we anticipate these studies using pre-clinical models of disease will identify new prognostic and therapeutic biomarkers including microbial mediators for IBD, arthritis and psoriasis.

Epidemiological studies indicate that IBD patients, particularly those with UC, have an increased risk of developing colitis- associated colon cancer (CAC). Therefore, the development of improved therapies for IBD is likely to result in decreased incidence of CAC.

How will you look to maximise the outputs of this work?

The outputs from this work will be maximised through the dissemination of our findings in publications and conferences. In addition, we will continue to publish our negative results (when relevant) in the hope of minimising redundant work.

We also have a number of successful collaborations in both academia and industry.

Species and numbers of animals expected to be used

- Mice: 59,100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate species for our studies because the mouse models that we will utilise are the best characterised models of gastrointestinal infection and inflammatory diseases, and they show the main features of human disease whilst being the lowest neurological sensitive species that does so. Most of our experiments will be performed on adult mice, but as microbial colonisation starts at birth and significantly changes in early life, we may alter microbial colonisation in juvenile mice. Very rarely we will use neonatal mice, where we need to alter development of the immune system before it is mature.

Typically, what will be done to an animal used in your project?

Many of the animals in this project will have an inflammatory disease induced, that will lead to inflammation in the colon, liver, joint or skin. Some animals will also develop colon cancer in the presence or absence of other inflammation. Disease induction usually involves injection or oral dosing of bacteria, bacterial parts, or a chemical, usually up to three times, but in the case of skin inflammation, daily application for up to 10 days at a time up to three times. Inflammation can take days or weeks to develop, depending on the model, but most experiments will be terminated within 2-6 weeks. Colon cancer takes months to develop so mice may be kept for up to one year to allow for tumour formation.

Prior to or during inflammation mice may also be injected with other substances such as blocking antibodies, antibiotics, or fluorescent dyes. Occasionally mice will be subjected to whole body irradiation followed by injection of bone marrow cells in order to understand the role of different genes and cell types. Animals will be allowed to recover for at least six weeks after this process before inflammation of any sort is induced.

What are the expected impacts and/or adverse effects for the animals during your project?

Following bone marrow transplantation, mice are expected to lose body weight and to experience general discomfort leading to symptoms such as temporary reduced social behaviour, loss of coat grooming and partial hunched posture. These symptoms are expected to last 3-5 days, depending on the treatment. However, full recovery is expected to be achieved within 7-14 days.

Injections are expected to cause local temporal pain, with no longer term effect.

Induction of inflammation is expected to cause inflammation at the site (intestine, joint or skin) leading to general discomfort. Mice can develop loose faeces, and lose body weight. Following onset these symptoms are expected to last 1-2 weeks in most cases. Mice that develop cancer (either spontaneously or following colitis) are expected to lose weight and may show temporary reduced social behaviour, loss of coat grooming and partial hunched posture. We do not expect tumours to reach a size where they impede normal biological functions.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the mice on breeding and maintenance protocols we expect the majority to experience subthreshold severity (approximately 70%), and a small number to experience mild severity (approximately 20%) and less than 10% to suffer moderate severity.

For the mice on experimental protocols, we expected the majority (approximately 90%) to experience moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Intestinal physiology and disease involves complex interactions between host genetic factors and the environment and these cannot be reproduced in vitro. A mammalian species is required to permit the study of the innate and acquired immune mechanisms that contribute to the development and control of intestinal infection and inflammation, and it is not possible to replicate the complexity or dynamics of these host-microbe interactions in vitro. Furthermore, the intestinal anatomy in which these host- microbe interactions occur cannot be reproduced in vitro.

Which non-animal alternatives did you consider for use in this project?

Where possible, some in vivo assays are replaced by in vitro assays to analyse immune cell function. For example, T cells can be grown in vitro under different conditions to manipulate their cytokine production and measure responsiveness to various stimuli. We have also established a 3D organoid culture model whereby different cellular populations are grown together in a gel matrix to re-create certain parts of the intestinal structure in vitro. This allows us to grow, manipulate and analyse some rare intestinal cell populations in the dish.

Why were they not suitable?

Although we are able to culture immune cells and bacteria in vitro, the conditions and media used to culture mammalian cells and intestinal bacteria are often mutually incompatible, meaning it is not possible to study interactions between them in a dish. Three-dimensional and co-culture systems are not yet advanced enough to replace all animal work.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated numbers of mice to be used are based on our experience running an integrated project over the last 25 years. The large numbers of mice for breeding genetically modified or harmful mutant mice reflects the use of these mice as donors of immune cells as well as hosts for immune cell transfer or infection. There are several very complex genetic crosses that mean that many mice need to be bred in order to get sufficient numbers with the right genetic mix, and we have accounted for this in our breeding estimates. In addition, to reduce the possibility of genetic drift arising due to inbreeding, where impact would be significant we will backcross genetically modified strains to the parent strain approximately every 10 generations. This will generate an increased number of animals to re-cross to a homozygous background.

We have also estimated the statistical power and frequency of our experimental set-ups to calculate the number of mice we will use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In experiments designed to test whether a particular manipulation has led to a reduction in the severity of disease compared to the control group, the number of animals in each group has been calculated to ensure a 90% chance of detecting a change that is statistically significant at the 5% level. For example for categorical data where the probability of mice in the control group getting colitis is 0.8 and where we would like to be able to reliably detect a reduction in the test group to 0.3, power analysis indicates a group size of 20. This group size, taken together with the number of different types of manipulation has been used to estimate the number of animals to be used.

Age and sex-matching within experiments will be used to reduce inter and intra-group variation, thus reducing the numbers required to generate statistically meaningful results. Where possible we co- house experimental groups in the same cage to reduce variability further, but this is not always possible when investigating the effect of intestinal microbes, as each mouse in a cage will share a very similar set of microbes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Every effort will be made to reduce the number of mice as follows:

- Use of pilot experiments with up to 5 animals per group for dose determination or model development, and up to 10 animals per group to determine intra and inter group variation.

- Inclusion of colonoscopy allows individual mice to be followed throughout disease development, both for IBD and cancer studies. This will reduce the number of mice required to look at disease kinetics and tumour progression.

- Maximal use of harvested cells and tissues. For example, spleen cells isolated from genetically modified mice may be used as a source of immune cells for in vivo transfer as well as for biochemical analysis in vitro.

- Mouse requirements will be reviewed regularly to avoid unnecessary breeding and breed only the animals that we need.

- Use of cells and tissues from shared control animals by multiple researchers.

- Archiving of frozen tissue samples to permit analyses of novel factors without additional in vivo experiments. We are creating a tissue bank of 10 different tissues from experiments using rare strains or mice lacking all microbes.

- Embryo freezing of strains not in current use.

- For xenograft experiments we will inject control cells and tumour cells into different flanks of the same mouse to allow an internal control and halve the number of animals that need to be used for each experiment.

- Where possible both genders will be equally used across experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models to assess the immune-microbial interactions that occur during infection and inflammation. We will therefore infect mice with microorganisms including bacteria and parasites, some of which will cause pathology in the gastrointestinal tract. We have carefully selected models of inflammation (in the intestine, skin and joint) that most closely mimic human disease whilst being minimally invasive to induce. Most of our models last 1-6 weeks, although our cancer models can last up to a year as it takes time for tumours to develop. For most experiments we are investigating the immune-microbial responses during inflammation, so once inflammation (or tumours) have developed our experiments end within 1-2 weeks. Occasionally we are investigating the repair response, as the diseases we are modelling are relapsing-remitting. In this case once inflammation develops we wait for spontaneous repair, which can start to happen within 2-3 weeks. We only perform repair experiments in models where we know that disease will self-resolve within 2-3 weeks.

As far as possible we have chosen models where we do not expect inflammation in any location other than the one of interest, e.g. a model of intestinal inflammation should not lead to concomitant joint or skin inflammation. One model of joint inflammation does also cause intestinal inflammation in a subset of mice (similar to human patients where a subset of ankylosing spondylitis patients develop additional intestinal symptoms), and understanding why this is the case and why it does not affect all animals is part of our scientific project. Many of our experiments are designed to assess the role of specific microbial or immune functions on disease outcome, and we expect that addition or removal of specific factors will in general ameliorate disease relative to controls rather than enhance it.

Why can’t you use animals that are less sentient?

The mouse is the most appropriate species for our studies because the mouse models that we will utilise are the best characterised models of gastrointestinal infection, human

IBD and cancer, and re- capitulate the main features of disease. The mouse is the lowest neurological sensitive species that shows similar intestinal and skin disease to the human.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To investigate the role of different cells and factors in intestinal inflammation and immunity, it is necessary to allow intestinal inflammation to develop to a moderate degree and the different disease characteristics mean that the end points will differ in different models. We have established refined monitoring schemes tailored to different procedures to assess the extent of clinical signs of IBD and to detect any adverse effects in experimental animals. We have shortened our models to reduce suffering to the shortest possible time, and our monitoring schemes allow us to end experiments as soon as practically possible after disease has developed. To accelerate tumour development, we will inject mice with the carcinogen azoxymethane (AOM), which does not impact on the induction of chronic intestinal inflammation, but accelerates tumour development allowing these experiments to be terminated earlier.

We use environmental enrichment and reduce social isolation of mice to a minimum by co-housing wherever possible. We make every effort to keep the number of procedures each animal undergoes to a minimum, for example by combining injections wherever possible. We also carefully consider the route of injection and where multiple routes are possible we use the least painful and invasive.

When possible and necessary, we use anaesthetics to reduce temporary discomfort during a procedure. Our preferred choice of anaesthetic is inhalation, which we use whenever possible. The equipment we use is very reliable to ensure the appropriate level of anaesthetic is delivered. When surgery is involved, we use appropriate aseptic techniques, monitor the animals before during and after the procedure. Most of our surgery will be performed under a terminal anaesthesia.

When using live pathogens, we use species that primarily infect the gastrointestinal tract, and avoid the use of species that cause more systemic disease.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We make use of the ARRIVE guidelines both for planning and reporting experiments, and we will also make use the NC3RS Experimental Design Assistant (EDA), G*Power2 and other online tools to assist with calculating appropriate experimental group sizes. If experiments are more complicated and require more specialist input we will seek support from statisticians local to our institute.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly attend animal welfare meetings. When utilising new treatments or models we will seek the advice of experts in the field to ensure that we are using the most appropriate techniques and monitoring. We use the NC3Rs website to keep up to date on more general advances in the 3Rs (https://www.nc3rs.org.uk).

26. Neuron-glia biology in neurodegenerative disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Ageing, Alzheimer's disease, Inflammation

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the role of glia and neuron-glia interactions in neurodegeneration, particularly in Alzheimer's disease

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Alzheimer’s disease accounts for around two thirds of all dementia cases (around 33 million people worldwide) with a complex cellular response and no treatments that can stop, slow or prevent it.

Understanding the biological mechanisms behind the disease is essential to finding ways to treat it.

What outputs do you think you will see at the end of this project?

The project comprises basic scientific research that will increase our understanding on the role of glia (support cells in the brain) in Alzheimer's disease and neurodegeneration in general. The outputs will primarily take the form of publications in peer-reviewed journals, but will also be disseminated at academic conferences and to the lay public via popular science initiatives. Materials, data and methods may, where appropriate, be disseminated online.

Who or what will benefit from these outputs, and how?

By understanding the role different brain cell types play in neuroinflammation we can begin to dissect out the different stages in Alzheimer's disease progression. In the short-term, the primary beneficiaries will be other scientists working in the field of neuroinflammation, ageing and Alzheimer's disease. In the medium term to long term, the basic science knowledge gained may have the potential for clinical translation. For example, understanding key pathways in disease progression may identify new targets for developing new pharmaceutical or therapeutic interventions in Alzheimer's disease.

How will you look to maximise the outputs of this work?

We will share data, analysis tools and resources between other members of our research community and this will reduce the number of replicated experiments in the field and the number that we need to do in our laboratory. In addition, we will disseminate our research by publishing results in peer reviewed journals. We aim to publish all results, including those that do not confirm our hypotheses. We will also present our work to academic peers at scientific conferences (national and international), and engage with public partners to disseminate our results.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will do these experiments in mice because of the number of available mice that are genetically suitable and the comparison with previous studies in the scientific literature. Using transgenic mice has the distinct advantage that we can use mice that express diseases, such as Alzheimer’s disease. We will use adult and aged mice, since these ages represent the most relevant for the study of age-related neurodegenerative diseases.

Typically, what will be done to an animal used in your project?

Some animals will be bred, and may have dyes injected to label the blood or cell types or drugs injected to alter the function of some cell types, and will then be humanely killed in order to study their tissues.

Some animals will undergo a surgery, where a small part of their skull is replaced with a glass coverslip to give us repeated optical access to the brain. Adverse effects after the surgery may reach moderate levels of severity for a short period of time, but all animals receive pain relief and are closely monitored until they recover completely. After that time, they will have a number of non-painful behavioural tests, some of which will also involve measuring from the cells in their brain using light. These tests may be repeated several times. At the end of these behavioural measurements, the animals will be humanely sacrificed and their brain tissue may be used for post-hoc analyses.

What are the expected impacts and/or adverse effects for the animals during your project?

The animals will undergo surgery of moderate severity. This means that the mice will be quiet and move less for a day or two after surgery. The animals will be given painkillers after surgery. Animals might lose a bit of weight, but will typically regain that weight within two to three days. Some animals will also be allowed to age (up to 24 months) meaning they may experience certain discomforts associated with the ageing process. At the end of experiments, or if mice show signs of ill health, distress or suffering that are not improved or resolved within a timeframe approved by the veterinary surgeon they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

30% of mice will experience a severity category 'Mild'

70% of mice will experience a severity category 'Moderate'

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice will be used in this project. Studying the cellular mechanisms involved in dementia and AD is extremely complex and involves understanding the interactions between different systems in the body (e.g. nervous, immune, and vascular). It is very difficult to mimic such complex interactions ex vivo, and whole animal in vivo experimentation is therefore vital in order to obtain a greater understanding.

The proposed studies could not be undertaken in lower species because they do not show such similarities to humans (e.g. they do not have similar glial cells), and in vitro experiments do not allow the study of interactions between different body systems (i.e. immune and vascular), which are critical for this project. Thus, the questions and hypotheses to be addressed cannot be fully studied in vitro alone and require in vivo studies.

Which non-animal alternatives did you consider for use in this project?

This project will be done in parallel to studies using non-animal alternatives such as Human post-mortem and brain biopsy studies.

Induced pluripotent stem cells (iPSC) Brain organoids

Why were they not suitable?

The use of non-animal studies will provide us with useful information that can be used to design our animal experiments. However, in vitro experiments (e.g. using induced pluripotent stem cells or brain organoids) do not allow the study of interactions between different body systems (e.g. immune, vascular and nervous) which are critical for the understanding of the physiological changes occurring in Alzheimer's disease. The use of human post-mortem and brain biopsy tissue, while helpful, will only provide us with a snippet of the cellular states at the later stages of the disease, but in order to understand disease progression we need to be able to study the brain at multiple timepoints during disease development in a living organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated based on previous experience when dealing with complex genetic crosses in mouse breeding as well as estimates on the number of experimental animals required based on expected effect sizes from the literature, past work, and in vitro pilot experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Several factors lead to a reduction of animal numbers, including reducing variation and good experimental design involving the use of appropriate statistics. In particular statistical tests will be used to ensure that we use the minimum number of animals possible to reliably interpret our data.

We will design our mouse breeding strategies carefully to minimize the number of generations necessary to reach the desired endpoint (i.e. desired combination of transgenes/ mutations), and to cut down the number of unwanted offspring. Where appropriate, we will use otherwise unwanted offspring for negative control experiments.

In our experimental design, we always collect as much data as possible from each animal. With longitudinal experiments, we repeatedly measure the same structures or cellular activity over a long period of weeks. This approach allows us to directly compare the structural/functional plasticity in the same synapses, cells and animal before and after sensory deprivation or alterations in behaviour.

Furthermore, in each animal, we can collect data from many cells, increasing the amount of data from each animal used and reducing the overall animal number necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding and holding lines as frozen down embryos and sperm will be used to minimise the number of mice being produced for these studies. Genetically modified lines will be sourced from repositories to avoid remaking of lines whenever possible. Any excess stock will be offered to other researchers to minimise wastage. Pilot studies will be undertaken to ensure the correct time course and experimental paradigms for our experimental purposes before larger scale studies. Tissues sampled from the animals used in this project will be shared with other researchers and the data produced linked to that generated by the project, to maximise long-term utility.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents have been chosen for this work as the least sentient species to mimic the human nervous system well enough for our work to be relevant to understanding of human brain function and disease. Mice are essential because the experiments require the use of the latest and most refined transgenic technologies to identify and label cell subtypes, to allow cell specific knock-in and knock-out approaches, to express genetically encoded indicators and optogenetic modulators and to model human diseases (e.g. Alzheimer's disease). These mouse models are not expected to exhibit any harmful phenotype.

We will be using chronic imaging in combination with cranial window surgery preparations, which allows us to follow the same cells over a period months. This preparation is ideal for examining how the same cells are changing their structure and function over time during disease progression. Cranial window surgery is necessary to allow us to image individual cells at a resolution that will allows us to draw meaningful results. This protocol minimizes suffering, as the initial surgery is the only one in which any pain may occur. All imaging sessions afterwards will only require light anaesthesia and no surgical intervention. For the surgical step appropriate anaesthetic/analgesic regimens and post- operative care will be used to minimise pain. Any surgical procedures will undergo regular review to identify further refinements to minimise animal suffering including optimisation of implants and anaesthesia/analgesia regimens.

In addition to chronic imaging, we plan to extract tissue from mice for in vitro downstream applications. These terminal experiments will take place entirely under anaesthesia and therefore will have minimal suffering. We plan to use these experiments to test a number of potential mechanisms before testing them in vivo, which will help reduce the number of animals undergoing the in vivo paradigm and that may suffer as a result.

Why can't you use animals that are less sentient?

Rodents will be used for this project as they represent the least sentient species appropriate for this type of work. Other species which are less sentient (such as invertebrates) cannot be used because they do not show such similarities to humans (e.g. they do not have similar glial cells which form the basis of this proposal). Mice which are genetically tractable, allowing transgenic identification and manipulation of specific cell types and there are a significant number of well characterised transgenic mouse lines available to model disease.

Animals at a more immature life stage cannot be used because this work focuses on the cellular physiology of ageing and age-related disorders and as such, we need to work in adult animals.

A number of our experiments are done in animals that have been euthanised or are terminally anaesthesised, but in order to look at long term cellular changes throughout disease progression we need to carry out some chronic experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animal facility staff run a comprehensive health-monitoring programme whereby animals health and welfare are checked and recorded on a daily basis. The animals will be maintained under conditions where their health status can be protected as far as is reasonably practicable. We use refined holding techniques for the animals, as well as group housing and enrichment. We use appropriate anaesthetic/analgesic regimens (including pre- and post-operative analgesia) to minimise pain and will refine these with advice from the NVS to ensure that we are using the best possible option given our experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Resources hosted on the NC3Rs website, in particular:

- ARRIVE guidelines on experimental design and reporting results.
- PREPARE guidelines for planning animal experiments
- 'Procedures with Care': 'Aseptic Technique in Rodent Surgery'.
- Rodent housing and husbandry
- Rat and Mouse Grimace scales

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs from several sources:

- Liaison with our animal care staff and NC3Rs representative
- Technological advances in the published scientific literature
- The NC3Rs website

27. Immune-based treatments for cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Therapy, Immune, Microenvironment

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will characterise the immune cells found in cancers and study how immune pathways can respond to cancer treatments. This will help to identify new ways of treating cancer by working out which types of cancers or which treatments respond best to immune-based cancer treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how cancers and immune cells interact has led to the biggest breakthrough in anti- cancer treatment in recent years. Despite this, most people with cancer don't

benefit from these treatments because they only work for a minority of cancers. It is interesting that most cancers where immune-based treatments don't work well are the same cancers which are caused by long-term inflammation. We have previously found immune pathways involved in inflammation to be active in aggressive cancers. We can alter these pathways using lab techniques or using treatments, and then study how cancers change their behaviour in response to these alterations using mouse models. Using mouse models means we can study these pathways in cancer in the context of an active immune system and response. This is very important as many different aspects of the immune system influence how cancers behave, which we can't model using cells in the lab. There are already drug treatments available targeting some of the pathways we are interested in, and knowing who could benefit from these treatments is important.

What outputs do you think you will see at the end of this project?

Cancers that are aggressive are typically inflamed. This makes them resistant to current cancer treatments. We have identified some ways in which this inflammation could be tackled by studying immune pathways in cancer cells. In this project we want to further development understanding into the role of inflammation in cancer development, spread and response to treatment.

We already work with international groups (in the US and Europe) in this area. We anticipate this work will be published in journals freely available to the wider community (called "open access"). We will also present this work at national and international scientific meetings. We work with patient groups to inform our research and make sure it stays relevant to patients and their needs.

We anticipate we can identify new treatments as a result of the work described in this licence. We also expect we can improve our understanding of which kinds of cancers respond best to combination treatments. Promising results from this work identifying new combinations or ways to tailor treatments will be brought forward to clinical trials.

Who or what will benefit from these outputs, and how?

In the short term, the scientific community will benefit from deeper understanding of inflammatory pathways in cancer. These short term outputs will primarily be publications and presentations within the scientific community.

In continuing our collaborative approach, we anticipate an ongoing acceleration in sharing techniques and refining approaches between our group and others.

In the longer term, we anticipate impact in new combination treatments or better selection of patients for existing anti-cancer treatments. These may not be evident in the five year span of this project as typically these treatments and selection techniques can take up to 10 years to get to the clinical setting.

How will you look to maximise the outputs of this work?

The licence applicant has demonstrated a collaborative approach in the development of this planned project, and has established collaborations both locally and internationally which will accelerate the development and strengthen the analysis of findings from this project.

Findings will be shared using open-access papers. Any negative data will be published to prevent unnecessary duplication of approaches taken during this project.

Species and numbers of animals expected to be used

- Mice: 3750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have demonstrated from human studies the profound effects of tumour-driven inflammation on the immune response to cancer, and effectiveness of anti-cancer treatments. We will use adult mice to study these pathways in established cancers and in cancer spread. By using mice, we can manipulate these pathways in the lab prior to injecting cells to better understand the impact of immune pathways on cancer development and spread. We can also use adult mice to study responses to cancer therapy and in particular, immune responses to specific treatments. We use mice with an active and intact immune system in this project to help us answer these important questions. We use adult mice as they have a mature immune system.

Typically, what will be done to an animal used in your project?

We will inject cancer cells in the mice, sometimes just under the skin and sometimes through a vein that results in lung disease. In some cases we will inject cancer cells directly into the abdomen. Using these approaches allows us to represent human cancer in the mouse. We can then use genetic alteration of immune pathways, or immune or anti-cancer treatments, to study the effect on tumour growth. We can specifically study immune cells in the tumour to under this better.

Other injections animals might receive include injections into a vein to allow us to take images of tumours using a scanner. They may also receive injections in the stomach, into the veins or under the skin which are anticancer treatments. In some cases we will inject these directly into the tumour.

Surgical procedures on mice will not be conducted in this work.

When we take images of the cancer, we will usually give the mouse a mild inhaled anaesthetic, which we expect will keep them still and comfortable without causing distress.

We may take very small amount of blood samples from the mice, usually from the tail vein, up to a maximum of 4 times.

Most experiments in this project could be measured in terms of weeks - we do not expect we will have mice on treatment for more than 3 - 5 weeks in total. In some cases, where mice recover fully from the cancer as a result of treatments given, these mice may stay alive for another few weeks to allow us to check the immune system responses to cancer. This involves an injection of cancer cells to the back or side of the mouse which will be permitted to grow to only a small size.

What are the expected impacts and/or adverse effects for the animals during your project?

Most mice on this project would be expected to develop cancers. They may have this cancer as a lump on the skin or breast area, or in the lungs, for a number of weeks. We expect most animals will not experience serious discomfort. In the protocols described in this project we have identified conditions that would mean that we end the experiment early to prevent the mice experiencing suffering.

Some mice may receive additional injections of anticancer treatment, injections to enable imaging of the cancer, or radiotherapy treatment. We have carefully controlled the dose or number of treatments a single mouse would receive. Mice receiving these treatments would only receive them for a few weeks in total.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most protocols in this licence are of moderate severity as they involve the induction of tumours and study of cancer. Out of all the animals on this licence, most would experience a moderate severity as they would develop a tumour, undergo imaging and receive treatment; or receive an intravenous injection of tumour cells which results in tumours in the lung; or receive radiation or systemic anti- cancer treatments. For all mice, we expect 75% on protocol 1 will experience moderate severity, up to 95% on protocol 2, up to 98% in protocols 3, and 4 and 40% on protocol 5. We would expect other animals to experience a mild severity. No protocols on this license are "severe". Typically induction of a tumour and receiving a treatment are considered "moderate".

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While we can study some pathways and effects using cells in the lab, the immune system in particular is complex and cannot be recapitulated outside of an animal model. A cancer is composed of multiple cells types - cancer cells, immune cells, blood vessel cells, fibrous cells and the extracellular matrix or secreted substances. All these factors within a tumour contribute to the immune response, and currently it is not possible to model this complex interaction outside of using an animal model such as mice.

Cancers often spread in humans, and this is ultimately what causes death from cancer. Using animal models to study this spread is very useful in trying to develop techniques and treatments to prevent this spread, and therefore aim to reduce deaths from cancer.

We do extensive work on the lab bench, using cells and co-culture models (where we grow two or more different cell types together such as immune cells and cancer cells) to make sure our experiments in animals are streamlined. We wish to use a minimum number of animals to answer what are essential questions about how cancers and immune cells interaction.

Only the use of mice will allow up to study the multiple features of cancer and allow the capacity to test potential future treatments.

Which non-animal alternatives did you consider for use in this project?

A literature review of current immunology methods was performed, with a focus on up-to-date development of human explant and organoid cultures. Explants and organoids are examples of techniques that take tumour samples donated from patients and grow them in the lab setting. We can also use blood samples from volunteers. In addition, we are studying the immune cells in cancers by using specific markers to stain and measure them. Where possible we will use these donated samples. Where these do not address important questions, we will use animal models to study more complex questions.

Why were they not suitable?

These human samples of tumour do not contain the full components of an immune system (for example, lymph nodes) and are unable to bring new immune cells in to the cancer. These human samples can typically only be maintained for 2 weeks, and don't permit the study of cancer spread or meaningful response to treatment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our estimations are based on our previous knowledge on the variability of the outcomes of interest, for the specific animal models used in our lab or from collaborators.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use a variety of strategies to ensure that the minimum number of mice necessary are used in each experiment. We use the NC3Rs experimental design guidelines to design our experiments, for example by ensuring randomization and blinding to avoid bias and increase reproducibility. We use data from small pilot experiments to help calculate animal numbers.

We use standard operating protocols in the lab, meaning that we expect that our care of cells in the lab, the injection of cells, the preparation of drugs and administration of these drugs to mice, all meet the same standard and are consistent. This means that we can design robust experiments using the minimum amount of animals.

We use appropriate controls - for example, control animals do not receive the anti-cancer treatment but receive an injection of fluid so that nearly every condition is the same. By changing only one condition in each group of animals, we can study the effect of that one condition closely.

We house and handle animals using standards that are consistent. We use appropriate animals housing, appropriate protective clothing, and ensure consistency in our approach.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For clinical interventions, such as testing anti-cancer agents, pilot experiments will help us to gain understanding of the response to therapy and statistical variability.

The minimum number of mice that will give a meaningful result will be determined using power calculations. For each animal treated or included in an experiment, we will harvest tissues and blood from the sample animal to obtain as much information as possible. By using imaging, we can follow the same animal over time, which gives meaningful results and reduces the need for additional animals to be studied.

Experiments testing novel treatment combinations will be randomised to ensure variability in tumour growth does not affect experimental outcome.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The least sentient mammalian species that is appropriate for this work, namely mice, will be used for this project. The availability of gene knockout and transgenic mice as well as the ability to manipulate mouse cancer cells in the lab using gene editing techniques gives a great deal of usefulness to the study of cancer in mice. We use good laboratory practice, high standards of training and project management to ensure we continually review our procedures and keep up to date with available refinements.

Why can't you use animals that are less sentient?

In order to study the interaction between a mature immune system and cancer, adult mice are the preferred model. Mice are the most commonly used species for the types of studies we are proposing in this project. Mouse models have been extensively used in the development of a wide range of chemotherapies and immunotherapies alone, and in combination with radiotherapy. They are classified as the lowest order species in which we can study tumour growth and adaptive immunity in a way relevant to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For each step in the protocols we have introduced monitoring and controlling measures to minimise the harms for each animal used in this licence. Some of the most significant ones are specified below:

Tumour induction and growth: Tumour induction will be performed under brief recovery anaesthesia, which allows precise injection of tumour cells to minimise the risk of cancer cells invading muscle.

Tumour cells will first be tested in a small number of animals to analyse expected growth curves and optimise conditions of tumour formation. Only tumours models which are useful and appropriate will be used in further experiments.

Administration of substances: We will typically use substances that are known to be non-harmful at the appropriate dose.

Radiation: Radiation will be applied locally to the superficial tumour, under anaesthesia. Damage to non-target tissues is minimised by the use of lead shielding or focussed

radiation (including imaging) and precise positioning under anaesthesia, or by the use of a linear accelerator (FLASH-RT) that spares normal tissue. We will use FLASH-RT as a means to reduce the side effects of irradiation on normal (non-cancer) tissue. This is in part due to a reduction of the time needed to deliver the irradiation, from minutes (X-ray radiation) to fractions of a second (FLASH-RT).

As a general measure, we will continuously revise our methodology and adapt it according to new policies, technologies and approaches aimed at minimising welfare costs. To achieve this, we will attend the seminars provided by the NC3Rs, keep up to date with NC3Rs recommendations via its website and communicate with the Regional Programme manager for advice to improve the 3Rs in our experiments. Together with the University 3Rs Committee we will diffuse our particular advances on the 3Rs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

A number of approaches will be used throughout the project to minimise adverse effects. Published guidelines for best practice will be followed: for example, Laboratory Animal Science Association Good Practice Guidelines Series 1/Issue 1) October 1998. Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit) and Collection of Blood Samples (Rat, Mouse, Guinea Pig, Rabbit).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Those licensed to work on this project will sign up to the 3Rs mailing lists (if not already signed up). To expand our knowledge on advances in the 3Rs, we will keep up to date with 3Rs newsletters, prepared by the University 3Rs subcommittee and by attending the 3Rs Research day. We will also attend the seminars provided by NC3Rs, keep up to date with NC3Rs recommendations via its website and seek advice to improve the 3Rs in our experiments.

28. Cell fate determination and morphogenesis during early mammalian development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

embryo development, cell movements, stem cells, cell differentiation, morphogenesis

Animal types	Life stages
Mice	embryo, pregnant, adult, juvenile, neonate
Rats	embryo, pregnant, adult, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our broad aim is to understand at the cellular and genetic level how embryonic pattern is specified. We seek to clarify which cells give rise to various organs in the embryo, how cell movements generate the shapes of these organs and how genes control these events during early embryonic development. We will achieve this by:

Characterising the movement and fate of cells of the embryo, from pre-implantation to early organogenesis stages

Elucidation of the molecular and cellular control of embryonic morphogenesis

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Large-scale cell and tissue movements play a major role in shaping the mammalian embryo. For instance, the embryonic region that gives rise to the heart actually starts out in front of the region that forms the brain. Little is currently know about how the movement of cells and tissues is coordinated to give the intricately shaped structure of the body and how our genes control these complicated movements.

These questions are intrinsically interesting as they address the issue of how we (with a distinct front and back, organs of various shapes etc.) develop from a fertilized egg that is roughly spherical and has no features distinguishing any one region from another. They are also medically important as congenital abnormalities such as various heart defects, cleft palate, spina bifida etc. are the result of improper cell or tissue movements during development. Understanding which populations of cells give rise to different portions of these organs may help in preventing or treating such disorders. This knowledge may also identify stem-cell populations in the embryo or adult that could be used treat various conditions in humans.

What outputs do you think you will see at the end of this project?

Publications, that will advance our understanding of the molecular and cellular mechanism that control embryonic development. All publications will be open-access. The findings in these primary publications will in time be included in text-books and therefore contribute to teaching material in the biomedical sciences.

Public repositories of data that will be of use to other scientists, and occasionally, to the lay public. This will include, but is not limited to: single cell transcriptomic data; single cell epigenomic and genomic accessibility data; time-lapse movie repositories of embryonic development

New methodologies and software for analysing data, both nucleotide sequence as well as image data. The former might include approaches for interrogating transcriptomic data and the latter will include tools for automatically detecting cells outlines. These computational tools will be made publicly available on repositories such as GitHub.

My group is active in outreach activities. For example, we have participated in the annual Science Festival for the past five years. We incorporate our research findings into our outreach material.

We use time-lapse recordings of embryonic development to explain our research to members of the public. We also use our high resolution confocal image volumes of embryos to print 3D models of mouse embryos and embryonic hearts, so that the public can appreciate the remarkable similarities between mouse and human embryos. This new

findings resulting from this project will similarly contribute to addition material to enhance our outreach efforts.

My group is active in public engagement activities where we work closely with non-scientists over a longer term. For example, we have collaborated with a dance group, that uses dance as a medium whereby disabled young people can express themselves. The young dancers used our time-lapse volumes of heart development as inspiration for the dances they designed. the dances they designed. Similarly, the research outputs of this PPL application will be used as inspiration in our future engagement with other artist and members of the public and will likely result in the production of works of reflection, self-expression and art.

Who or what will benefit from these outputs, and how?

In the short-term:

The scientific community will benefit from the knowledge we generate. It will help advance or collective understanding of fundamental cellular processes relevant not only to the embryo but also the adult.

Other scientists will be able to use the knowledge we generate to design their experiments to advance their areas of study.

Other scientists will benefit from the data (e.g., transcriptomic and epigenomic data) we generate, which they will be able to directly interrogate or compare with data from their system.

Researchers working in the area of directed in vitro differentiation of stem cells will particularly benefit as the understanding of normal cell specification that we generate will enable them to design a rational approach for generating in vitro different cell types of choice.

These outputs will be used by other scientists in their research and will therefore potentially contribute to the development of regenerative approaches to treat ailments.

Lay members of the public with an interest in science will benefit from those aspects of our research findings that we disseminate through our public outreach and engagement activities. This will help sustain public curiosity in fundamental research as well as well as respond to curiosity around the fascinating process by which we are shaped in the womb.

In the longer-term:

Our work focuses on fundamental biology. It provides the foundations on which new therapeutic approaches can be developed for clinical impact.

Understanding how normal development occurs will enable us to understand how congenital abnormalities arise. In the longer term, this will help us to avoid or, potentially, even treat them.

How will you look to maximise the outputs of this work?

All our publications will be in Open Access journals, of be made Open Access

We will deposit pre-prints of our manuscript on bioRxiv prior to submission to journals for peer- review.

Where possible, we will create easily usable web-sites that others can use to interrogate our data with minimal technical background. As an example please see: https://marionilab.cruk.cam.ac.uk/heartAtlas/

We will make computation tools we develop easily accessible, either through GitHub or through university repositories. As an example of this, we developed a tool for quantifying protein levels from image data of mouse embryos. This tool is freely available from https://process.innovation.ox.ac.uk/software/p/13299a/silentmark-academic/1

We will publicise our results at conferences through oral presentations and posters.

Species and numbers of animals expected to be used

- Mice: 35,600
- Rats: 700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodent embryogenesis is similar to human embryogenesis at the genetic and cellular level, and therefore represent a viable model for experimental studies of human embryogenesis. The knowledge we gain from experiments on mouse embryos is essential in understanding the development of human embryos.

Large-scale cell and tissue movements play a major role in shaping the mammalian embryo. For instance, the embryonic region that gives rise to the heart actually starts out in front of the region that forms the brain. Little is currently know about how the movement of cells and tissues is coordinated to give the intricately shaped structure of the body and how our genes control these complicated movements. The aim of this project is to understand at the cellular and genetic level the movements that shape the body. We seek to clarify which cells give rise to various organs in the embryo and how cell movements generate the shapes of these organs.

We will use rodents (mice and rats) as a source of embryos for the experiments we need to perform to address the above questions.

Typically, what will be done to an animal used in your project?

The majority of animals produced under this licence (no more than 36,300) will be used for breeding to maintain strains with specific genetic characteristics and for embryo production. Up to 1% will be recorded as having experienced a mild procedure. All other animals will experience little or no effects from the breeding programme, referred to as sub-threshold.

A number of animals will undergo surgical procedures. No more than 1000 female mice will receive embryo transfers and no more than 100 male mice will undergo a vasectomy. These animals will undergo one procedure with full recovery expected without incident. The surgical procedures are needed to produce new genetically modified mouse lines.

A number of females (no more than 2000) will be used for the production of early embryos using a hormonal injection procedure termed super-ovulation. This is performed to obtain embryos for experimentation or for long-term storage/archiving (cryopreservation).

A number of animals will receive one or more injections of substances that induce an effect in the cells of that animal, or in the cells of embryos females are carrying (no more than 5200).

What are the expected impacts and/or adverse effects for the animals during your project?

Since we study embryonic development, the vast majority of our animals (~90%) are used primarily as a source of embryos and therefore are simply maintained, mated and then killed so as to collect their embryos for experiments.

We will breed and maintain wild type and genetically modified animals. The vast majority of mutants will have nil or minor/transient adverse effects. Genetically modified animals of greater severity (e.g., mild limb defects such as digit duplications, tail defects such as short or entirely absent tail, tooth defects such as loss of specific teeth) will be bred on a separate moderate protocol.

The only procedure the majority of our animals will be subject to is an ear biopsy and/or an injection, which is of mild severity.

Superovulation procedures are expected to result in no more than transient discomfort and no lasting harm.

Surgical procedures will be carried out aseptically. In the unlikely event of post-operative complications, animals will be killed. In the case of wound breakdown, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery. Animals are expected to make a full recovery from the anaesthetic within two hours.

For any application of substances done under protocols in this license application, any transgene inducing agents normally have very little or no adverse effects on the overall welfare or health of the animals. In extremely rare cases, adverse effects may arise in the mother from the expression of genes in her foetuses, induced by the administration of substances. There is a very low risk of harm to the mothers from the method of delivery of substances (<1%).

The majority of animals will be killed humanely at the end. Those that are not killed will be transferred to other authorised scientists who might be interested in studying them.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold 89%
Mild 1%
Moderate 10%

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objective of this proposal is to further our understanding of mammalian embryonic development. Since so little is known about this process, it has to be directly studied in embryos from mammals.

Amongst mammals, research using the mouse comes with a relatively low ethical cost. The mouse is also the most appropriate mammalian model due to the well-established molecular genetic technologies available for experimentation.

The majority of developmental stages that we focus on in this project are early enough to not be covered by ASPA. Our use of the embryo to model basic cell biological processes that are also important in adults, such as cell migration, can be considered replacement as otherwise, one might have to use protected animals to study these processes.

Which non-animal alternatives did you consider for use in this project?

Mathematical models
Tissue and cell culture models

Why were they not suitable?

Mathematical models of development cannot entirely replace experiments on embryos since the models are themselves founded on experimentally obtained knowledge and are only as good as the information that went into building them. Therefore, while computational models can help identify the most plausible of several hypotheses for testing experimentally, they are limited in their ability to tell us completely new things about embryogenesis. Therefore, to understand the processes outlined in this project, we need to perform experiments on actual embryos.

Well-constructed models however show 'emergent' properties that were not explicitly programmed into them. We have a continuing collaboration with a group of mathematicians to use mathematical models of cell movements in our studies. This will enable us to use the model as a replacement for embryos in specific initial experimental investigations. It should be noted however that results from the use of mathematical models will invariably have to be verified using actual biological samples.

The specific tissue interactions during embryonic development cannot be accurately recapitulated in cell culture. In recent years, several in vitro 'embryo-like' models derives from ES cells have been developed. Comparison with known developmental events suggest that these do not faithfully recapitulate known aspects of in utero development, particularly with respect to the role of extra-embryonic tissues in patterning, which is a major component of the research in the project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of animal numbers is based on the following considerations:

the number of embryos we will require to address the objectives of this project and the average yield of embryos at various stages from females of a variety of backgrounds (including, but not limited to CD1, C57Bl/6, 129 and Balb/c)

a forecast of various genetically modified mouse lines that we will need to generate, and the numbers of animals required to generate such transgenic lines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to reduce animal use, we are developing mathematical models that will help us to rule out certain experiments using computer simulations.

A thorough literature search is conducted to ensure that experiments that are undertaken do not duplicate reliable data that is already published or present in data/resource repositories.

Principles of experimental design are applied in order to minimise the number of experiments required to achieve reasonable statistical significance and data reproducibility.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When setting up crosses to produce embryos, where possible we use males homozygous for the reporter so that all the resulting embryos carry the reporter gene and are usable.

Where possible, instead of establishing permanent transgenic lines and maintaining a colony, we will use high-efficiency CRISPR technology to create ‘transient transgenic’ lines that will produce enough mutants for our experiments with fewer mice. This may include the administration of transgene inducing agents to pregnant dams that have undergone an embryo transfer in protocol

Protocol 1 and 8 allows us to produce a greater number of viable embryos from a single dam.

Where possible we will import previously generated GM mouse lines from colleagues/collaborators/resource centres.

Stock levels of mouse strains will be set to minimise animal breeding whilst at the same time ensuring that given strains are not lost. Trained animal house staff will carry out the majority of breeding and maintenance, constant contact and instruction will ensure the colonies are maintained at the correct levels.

To avoid unnecessary breeding/maintenance of strains we will archive (embryo/sperm cryopreservation) when no longer needed, where we can access them at a later stage. We will supply cryopreserved embryo/sperm to other investigators on request in order to reduce duplication of animal experimentation.

Where possible, we will use embryonic stem cell derived ‘embryoid bodies’ (EB) as a replacement for actual embryos. Our preliminary experiments suggest that we may be able to test hypotheses initially in EB before verifying results in actual embryos. This will lead to a reduction in the total number of embryos (and therefore animals) used.

Tissues/embryos, DNA/RNA and protein samples obtained from experimental animals/embryos are preserved, usually by storing in liquid nitrogen or at -80c, to allow where scientifically justified, use in other experiments by ourselves or other scientists who may request such reagents.

Where possible, we will harvest rat serum for embryo culture from our rat colony. Such serum will be used to verify the suitability of commercial serum for embryo culture. This will enable us to ensure embryo culture conditions are robust, thus ultimately leading to a reduction in the total number of animals used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project focuses on understanding mammalian embryonic development. The genetic basis for the development of mouse embryos is sufficiently similar to that of humans for it to be a viable model of human development. The majority of mice will be used only as a source of embryos, and therefore will not suffer any experimental procedures. The mice will be killed humanely in order to recover embryos from them.

Regarding specific procedures:

Vasectomy – Animals are expected to suffer no more than moderate harm due to the nature of the scrotal sac method which is the most refined method for this procedure. When available, we will acquire genetically altered or naturally sterile males that don't require vasectomy.

A surgical procedure termed laparotomy is routinely carried out on pseudo-pregnant recipient female mice (after mating with sterile males) for the implantation of embryos during production or rederivation of genetically altered animals. The procedure can be performed on one (unilateral) or both (bilateral) sides of the abdomen depending on the number of embryos requiring implantation, the stage of development at implantation and the number of pseudo-pregnant recipient mice available. An alternative non-surgical procedure termed transcervical transfer can be carried out but only on embryos at a specific late stage, referred to as blastocyst.

Superovulation by hormone injection is the standard method to collect the most oocytes and zygotes from the least amount of females. The appropriate concentration of hormone is age, weight and strain dependent, having been optimised in studies over many decades.

Under breeding and maintenance of genetically modified mice, most animals are not expected to suffer any more than mild pain. Expected actual severity is sub-threshold. Some animals are bred under a moderate severity and although not expected to show a harmful phenotype this is always a possibility. Adults that do show any harmful effects will be culled.

Administration of substances will be carried out by the most refined method available.

Why can't you use animals that are less sentient?

Non-mammalian species such as the zebrafish are not suitable for this work as they do not show several features of development seen in mammals (such as implantation in the womb, or a four- chambered heart). Moreover, mice are the lowest mammalian species in which it is relatively easy to make the genetic modifications required for this project.

The genetics of mouse development have been long studied, providing a good foundation for our work. Mice are the most suitable mammalian species of the lowest sentience that it is relatively easy to make transgenic modifications in. Technologies exist for knocking out gene functions, and chemically inducing gene expressions. The vast majority of the mutants we work with are recessive, i.e., they suffer no physical changes when kept as breeding colonies. In the homozygous condition they are generally embryonic lethal, so in general we do not maintain homozygous affected individuals for the majority of our mutant lines.

The genetic basis for the development of mouse embryos is sufficiently similar to that of humans for it to be a viable model of human development. Furthermore, the early mouse embryo is a powerful model for studying basic cellular and genetic processes that operate in adult contexts, both in healthy and pathological situations. One of our main research questions is how cell migration is controlled in epithelia. The early post-implantation mouse embryo is a good model for characterizing such movement by volume imaging as it is small and the epithelial tissue of interest on the surface of the embryo. It is also a good model for addressing the mechanistic control of cell migration, as it is amenable to manipulation in culture and various mutants with defective cell migration exist. In order to perform comparative studies on the influence of basic physiological processes on embryological patterning (e.g., initiation of cardiac contractile activity), we will occasionally use embryos from Rats, that are a well-established model system, particularly for cardiac physiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We make use of all method which allows us to have a higher efficiency of targeted homologous recombination (e.g. CRISPR 2C-HR) during microinjection.

We will keep up to date with all surgical procedures and their refinements and will attend any relevant workshops.

Laparotomy surgery will cause moderate suffering. Non-surgical implantation through the cervix may cause distress if the handler is not as proficient but is not expected to cause harm. Animals are expected to make a rapid and unremarkable recovery from the implantation procedure.

Analgesic agents will be administered.

Animals to undergo surgery are given at least 7 days to acclimatise before procedure starts.

We will ensure that embryo culture condition are optimal at all times. To this end, if required, we will produce our own rat serum.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

Code of practice for the housing and care of animals bred, supplied or used for scientific purposes.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By regularly attending the NC3Rs days organised at the University.

Regular contact with transgenic groups within our institution allow us to update each other with advances in techniques and training.

Departmental animal welfare meetings held termly allow us to communicate any 3Rs implemented within our group and invited members including NACWOs and NVS ensure that any new advances are disseminated.

Attending the termly Gold Standard and Animal Welfare meetings during which advances in the 3R are frequently discussed.

29. Synapses in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Alzheimer's disease, Synapses, Microglia, Immune response, Genes

Animal types	Life stages
Mice	neonate, juvenile, adult, embryo, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how risk-factors modify neuronal and glial phenotypes throughout the life span of models of Alzheimer's disease in relation to normal function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The leading cause of death in the UK is now dementia, the most common form being Alzheimer's disease, accounting for 12.7% of all deaths registered in 2018 (i.e. the most

recent data available at the time of preparation;
https://www.ons.gov.uk/peoplepopulationandcommunity/healthandsocialcare/causesofdeath/articles/le adingcausesofdeathuk/2001to2018). Alzheimer's disease is characterised by increasing deposits in the brain, both inside cells (tau tangles) and outside cells (amyloid-beta plaques).

There are no effective treatments for the disease. Those that are currently in use only temporarily alleviate the symptoms, probably by increasing general awareness and concentration but do nothing to slow the insidious development of tau tangles and resulting cell death.

A large hole in our understanding is how the initial triggers of Alzheimer's disease, raising Amyloid-beta and its subsequent deposition, lead to tau tangles and neuronal cell death. Mouse models of the disease have been very successful in modelling the early Amyloid-beta component but they do not develop tangles or substantial neurodegeneration, unless the link is bypassed by the introduction of changes in the tau gene. However, such changes in the gene for tau have never been identified in Alzheimer's disease (but are relevant to other related diseases).

Our previous work indicates that mice have a very strong immune response, involving many of the genes associated with Alzheimer's disease. While in humans these are typically changes that cause loss-of-function, in the mice the expression of these genes is increased. We therefore believe that one reason for mice failing to develop the late stages of the disease is this strong immune response and we therefore intend to employ models that compromise this response by introducing changes into immune genes and also to combine other high-risk-factors, like obesity.

By studying such models, we will gain invaluable information on how the immune system interacts with neuronal function; how obesity interacts with neuronal and immune function; how obesity interacts with the immune system to affect neuronal function; and, crucially, how all these combinations interact in ageing and Alzheimer’s disease. This information is gained irrespective of whether combining these factors leads to the ambitious aim of developing a ‘Gold Standard’ model, whereby mice develop all stages of the disease but in the absence of changes in tau.

What outputs do you think you will see at the end of this project?

The outcomes of the project cover several levels from, 1. synaptic transmission and study of neurones (nerve cells) and glia (the immune cells of the brain) within brain tissue to 2. measurement and localisation of brain proteins and gene expression and 3. behaviour. Moreover by using the same mouse brain for several different tests the data can be correlated and the number of mice used minimised. Our key outputs are thus run in parallel, allowing us to study the brain from genes to cognitive function. Specifically, we examine gene expression (neuronal and glial gene specific; or using a genome-wide approach), neuronal and glial protein expression, synaptic function and behavioural

outcomes. We will study these in a number of mice that will reveal how risk-factors interact to modulate disease and normal function. Importantly, the results of one section is not dependent on the success of other sections and, therefore, the chances of useful data that advance our knowledge is increased.

1) Alzheimer's disease is usually a disease of old-age. However, this crucial aspect is often overlooked, with many studies focusing on young adult or middle-aged mice, at oldest. This project includes animals right up to end-of-life and will therefore inform us about neuronal and glial function in both normal ageing and its interactions with Alzheimer's disease.

2) TREM2 is a microglial gene with rare gene variants that substantially increase the risk of developing Alzheimer's disease. One such variant is a loss-of-function mutation, TREM2R47H which increases the risk of developing Alzheimer's disease by more than 3-fold; a similar increase to that caused by more common APOE4 variant. Studying the TREM2R47H variant in knock-in mice will allow us to understand how compromised microglia affect disease-related effects.

3) Obesity is a major risk-factor for developing Alzheimer's disease. Feeding wild type mice a high-fat diet will allow us to understand the effects of obesity throughout life within the wider population. Feeding the Alzheimer's disease model mice a high-fat diet will offer insights into how obesity exacerbates disease. It is important to include the WT group to assess whether any changes seen in the APPKI mice are simply additive or are interacting with the Alzheimer's pathology. It should be noted that feeding wild type mice a high-fat diet does not induce Alzheimer disease-like pathology; this was established under our previous PPL and at time of application, a manuscript reporting these findings is in preparation. Conversely, we may employ dietary restriction, which has been shown to improve both life-span and quality of late life.

4) A key step in disease progression which is missing from mouse models of Alzheimer's disease is the development of tau tangles. One explanation for this is that human Amyloidβ may not interact with mouse tau in vivo in the way that occurs in Alzheimer's disease We therefore will examine whether humanising the tau gene (Mapt) affects neuronal function in otherwise normal mice and then whether humanising Mapt results in progression to Tau tangles in Alzheimer's model mice.

5) All interactions of the above will also be combined. We will thus study obese Alzheimer's model mice with humanised tau, compromised microglial function (TREM2R47H).

Each stage of these experiments will inform us about those functions and their subsequent interactions. We may also find that this combination of risk-factors results in mice developing tau tangles and neurodegeneration without the introduction of mutations in tau. Such a gold-standard mouse would be crucial for the wider research community, allowing elucidation of how raising Amyloidβ levels and its deposition into plaques results in tau

tangles. It would thus also provide a much improved model in which to test potential therapeutics. However, this would indeed be the 'icing on the cake' and the proposed outcomes above are not dependent on the development of tangles, nor indeed of changes observed at any one stage of the project.

All the results from the experiments will be published in open access format in high profile journals as well as being presented at conferences to maximise the benefit of our findings to the wider scientific community.

Who or what will benefit from these outputs, and how?

While unlikely to be achieved within the lifetime of this project, the ultimate beneficiary is the community at large. With Alzheimer's disease already the leading cause of death and the incidence rising along with the ageing population, it is crucial that we understand this disease. While the health benefits are arguably the most important, the economic costs to the taxpayer should also be considered. The annual costs for caring for people with dementia is more than that for cancer and cardiac disease combined.

The broader neuroscience and Alzheimer's research communities, both at a basic and clinical research level, will also benefit. At the basic level, understanding how risk-factors interact will inform further research in the field. Along-side the Alzheimer's field, the research will inform physiological research: how does ageing affect neuronal function and brain’s immune responses? How does obesity affect these responses? If the combination of risk-factors successfully results in generating the 'gold-standard' mouse model, then all subsequent basic research will also benefit from having a much improved model. Moreover, this model would then be available to benefit clinical research, as confidence in the animal research would be much improved and lead to better translation to the clinic.

How will you look to maximise the outputs of this work?

The lab is highly collaborative. Many of our publications are from authors of multiple institutions both nationally and internationally. Active collaboration that are currently in place include co-supervision of a PhD student, funded by the Swedish Alzheimer’s Foundation; a long-standing active collaboration with a world-renowned geneticist from our institution; ongoing interactions with other local dementia researchers; and interactions with clinical research staff.

All research is published in peer-reviewed and open access journals. We also have a freely accessed web-based database, allowing anyone to search for genome-wide expression data from 5 different transgenic mouse models and across the lifespan. This will very soon be updated to include data from the improved APP knock-in mice.

Publication of unsuccessful approaches has been notoriously hard to achieve and is fraught with problems with assessing the cause of the failure: inexperience, incompetence, poor specificity of the reagents used, lack of power to detect small effects, or indeed that there is a genuine lack of effect. However, our recent funding to develop organotypic brain

slice models of Amyloid β plaque development has failed to reliably produce plaques and we are aware that other groups have similarly failed, making it likely that the results (or lack there-of) are real. We are therefore investigating the feasibility of a group publication, using the independence of the groups and differences in approaches as a strength to support the idea that organotypic brain slices do not make a good model for Alzheimer's disease and thus prevent further ongoing attempts (and use of animals), unless someone can see where our previous combined attempts may have failed and improve on those.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice offer the best model for Alzheimer's disease: one that provides an in vivo mammalian central nervous system without using non-human primates. We have explored using rats as a better model, but these offer little if any further correlations to the disease than mice do, but have some extra welfare requirements. Conversely, invertebrate models such as flies and worms (which have their appropriate place in neuroscience and Alzheimer's disease research) fail to provide the similarities to humans, particularly in terms of brain structures and mechanisms of synaptic transmission. Importantly, mice are very amenable to genetic manipulation and reach old age within the life span of typical research grants (mice typically live to 24-36 months of age; a typical grant is 30 (or rarely up to 42) months).

By the very nature of Alzheimer's disease, typically a disease of old-age, we need to study adult and aged-adult animals. However, there are situations when using tissue from embryos or neonates is appropriate. When such situations arise, we indeed use these and they are included in this licence application.

Typically, what will be done to an animal used in your project?

The vast majority of animals undergo no interventional procedures such as surgery or substance administration. They are, however, bred carrying genes that in humans cause familial forms of Alzheimer's disease. Once they are weaned from the mother at 3-weeks-old, they are group housed with their littermates in a slightly enriched environment (bedding, chew toys, houses) and aged to an appropriate age (anything from weaning through to end-of-life at 24-36 months-old), until they are removed from the cage to the laboratory and killed humanely.

Once the animal is dead, the brain is rapidly removed to keep the tissue alive and submerged in artificial cerebrospinal fluid. We can then cut the tissue and keep brain slices alive for the remainder of the day, allowing us to make electrical recordings from either single or populations of neurones. From the same brain, we can also get immunohistological (information about distribution and amount of specific proteins) or genetic data, allowing us to correlate all of these.

The aim of this project is to combine risk-factors for Alzheimer's disease, investigating either their singular or combined effects. Specifically, we will compromise the function of microglia (the innate immune cells of the brain) and/or make mice obese by feeding them a high-fat diet. This may exacerbate the Alzheimer's disease phenotype, providing crucial information on how these risk-factors modulate the disease and potentially offering therapeutic targets by which we can hopefully slow the progression of disease. Conversely, we may employ dietary restriction, which has been shown to increase life span and, moreover, quality of extended life.

A small subset of animals may undergo surgical procedures from which they recover. This is to enable us to delivery substances to the brain to modulate neuronal (or other cells) function. Such procedures can either be short (e.g. injection under anaesthesia) or long-term in which we fit a small slow-release pump, designed to fit under the skin at the back of the neck of a mouse, and will release substance over the following month.

Some of the animals that have been on a high-fat diet or have undergone the surgeries described above, may undergo behaviour testing. This is usually reward-based testing. For this, the animal has food withheld overnight so that it is motivated to retrieve a food or liquid reward, typically sweetened condensed milk. Animals then explore an open arena or a maze, and are assessed on their ability to remember either previously encountered objects or previously visited areas of the arena or maze. We usually opt for single-trial tests that last for 30 minutes at most but are typically a couple minutes in duration and include habituation to the arena as part of the test on the same day. Following testing the animal may then be returned to the home cage for further similar testing later in life or be killed for the above described experiments examining neuronal function, genetics and immunohistology.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the animals, even ones that possess genes that cause Alzheimer's disease and have been combined with risk-factors, never develop to stages where they display clinical symptoms . However, one of the aims of the project is to exacerbate the progression of these models and we would therefore predict that some of the animals in the study may develop a neurodegeneration that presents with the animal having slow responses, moving little in the cage with a hunched posture, piloerection as well as loss of weight. This is distinct from the general slowing seen as animals age. If this phenotype becomes apparent, the animal is always killed on that or the next day and the brain used in the

described experiments. Importantly, a collaborator has used in-cage behavioural monitoring to assess some of the crosses we will use, aiding us to monitor clinical signs prior to likely onset. Feeding mice a high-fat diet offers further considerations. We have experience of this procedure from our previous PPL and have an established monitoring regimen to check weights, blood tests for diabetes and body condition to ensure that animals are able to move properly.

Contrary to perceived outcomes, dietary restriction is not detrimental to animals. 60% food intake provides sufficient vitamin and mineral intake and actually increases life-span and, moreover, quality of that life (Fontana and Partridge. Cell 161:106-118, 2015).

Following surgery, we have excellent recovery rates. However, interactions with the Alzheimer’s disease phenotypes could add unforeseen complications. We have in place an established monitoring system to ensure that mice recover, both immediately from the surgery and are not developing unforeseen adverse effects caused by the administered substances.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

While our protocols have either mild or moderate severity limits, most of the animals (>90%) will not develop clinical symptoms and would have a severity rating of sub-threshold.

Some of the Alzheimer's model mice (~10%), particularly those that also have compromised microglia and are fed high-fat diets, may start to show some overt traits such as diabetes and may reach a mild severity. A very small proportion (<1%) may show neurodegenerative-like symptoms and be considered as a moderate severity.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While there are certain questions that can be answered very well using either computer modelling or cell cultures, these do not replicate the complex interactions of an intact

animal. Understanding how the brain's immune system, particularly microglia and astrocytes, interact with neuronal cells requires an intact brain, which cannot be modelled in vitro or 'in silico'. If we then bring in how Amyloidβ modulates these interactions and their effects on the brain, such modelling is nigh-on impossible. Using rodent models is the best option, as they offer a mammalian brain that in many aspects is similar to human (same neurotransmitters, analogous brain structures), are readily amenable to genetic modifications and allow us to perform other interventions that would otherwise not be achievable ethically in human studies. Importantly, being able to kill the animal and remove the brain at variable stages prior to natural death permits high-resolution analysis that cannot be achieved with the current technology for live patients.

Which non-animal alternatives did you consider for use in this project?

Cell cultures.

Induced pluripotent stem cell (iPSC) cultures from patients.

Computer modelling.

Why were they not suitable?

At the time of our previous PPL application, there was limited evidence that a 3D mouse neuronal culture could replicate some of the pathological features of Alzheimer's disease, however this not been reproduced by others, or indeed the same group.

iPSC cultures do, to some extent, achieve the pathological features of Alzheimer's disease. However, adult levels of Alzheimer's-relevant proteins such as mature forms of tau are not present until 2 years in culture (it is an admirable feat to maintain such cultures for so long!) and, importantly, these cultures still fail to retain the complex in vivo interactions of CNS immune cells, sensory inputs or other peripheral inputs.

Moreover, to date, there are no reports of them having developed tau tangles as a result of APP mutations and thus present with the same key limitation of the animal studies in addition to the limitations discussed above.

Computer modelling again is very limited in relation to Alzheimer's research. Indeed, it is limited for understanding normal function where the types of interactions between different cell types and proteins are only just being identified and cannot therefore be factored into simulations. However, we are currently exploring modelling Amyloidβ plaque growth to understand different deposition patterns.

In addition, in our last licence, we explored using organotypic brain slices, which maintain the basic neuronal connectivity of the hippocampus and retain all the cell types normally present. However, similar to independent trials by other groups, we were unfortunately unable to reliably model Amyloidβ deposition. Organotypic brain slices do, however, have

an ongoing role within the lab in understanding certain neuronal functions, particularly related to normal function.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we propose to use were determined by power analyses based on a mixture of experience with previous experiments that indicate expected variation and effect sizes, thus numbers of animals required to reliably detect a real effect. Importantly, the numbers of litters we will need in order to supply the sample sizes we need were calculated using our local Biological Services Unit animal costs calculator.

The sample sizes identified from such analyses are then scaled up by the number of different experiments we intend to perform. We also factor in attrition, particularly when we are studying the oldest ages.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The design of the experiment has been peer reviewed several times (e.g., for our previous PPL 70/8999, for successful grants and for peer-reviewed publications.). Important factors we consider include: the random assignment of cages to different treatments (for example control versus high-fat diet); performing experiments blind to genotype/treatment wherever possible and always analysing blind to genotype or treatment group; ensuring that the appropriate sample sizes have been attained for replicates within a given experiments; avoiding pseudoreplication, which is a common error in electrophysiology whereby multiple cells are recorded from a single animal and then treated as independent samples; we have a degree of repeating experiments by independent operators or blind- reanalysis by two independent operators; wherever necessary, covariates are factored into the statistical analysis so that the influence of other different factors can be considered (it should be noted that, using genetically identical mice raised in identical conditions, rules out many of the confounding factors that are common to human studies.)

Wherever possible we breed mice in colonies that limit the number of unusable genotypes. For example, when homozygous and wild type mice are required, we try to avoid using heterozygous x heterozygous crosses as 50% of mice will not be useable (other than for replenishing breeders). We would therefore have three colonies: two larger colonies, one with homozygous x homozygous mice, the other with wild type x wild type; and a third, smaller colony of heterozygous x heterozygous mice, which enables us to confirm that there are no differences in development and rearing of young between mutant and wild type mice. Thus, we largely avoid producing animals with unusable genotypes.

We have also started using both female and male mice whenever possible and this is the case for most of our experiments. There are some cases, however, where the female oestrous cycle causes substantial variation which can confound the experiment. One example is densities of hippocampal dendritic spines, the sites of many synapses on neurones. There is a >15% variation in density between pro-oestrous and oestrous phases and thus we would identify potential phenotypes in males first for these types of experiments and later determine whether a repeat using female at known stages of oestrous is required. While it is of course extremely valuable to understand the role of the oestrous cycle and to tease out effects that may be dependent on these variables, this is a subject in its own right, requiring many more animals and substantial additional time and resources. Interestingly, however, for many of the variables we are testing we find little if any difference between male and female mice and generally use both. Indeed, all female mice that are bred (and are of usable genotypes) are now used in experiments rather than culled and sex is used as a factor in our multivariate analyses.

The tissue from all animals are used in multiple experiments. A single brain is bisected into its two hemispheres, which then are processed in at least two, sometimes three ways. One hemisphere can be fixed for immunohistological examination (visual examination of the distribution of particular proteins), the other frozen for other protein or genetic analyses.

Alternatively, one hemisphere is sliced for live recording using electrophysiology, the slices from which are often shared between multiple experiments, increasing the output; with the other hemisphere fixed for histology. It is possible to snap freeze one hemisphere, slice the other for live recordings and also drop fix one of these slices for histology. Importantly, this approach not only reduces the number of animals but also increases the power of the experiments as we can correlate the different types of results within a single animal (see next section).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As discussed above, breeding regimes try to limit the numbers of unusable genotypes. Staff members involved in setting up breeding have attended accredited courses aimed at improving and optimising breeding regimes for mutant mice. Breeding records are maintained within a shared online system such that pedigrees can be traced and future generations planned.

Using both male and female mice means that the overall numbers of mice used in a given experiment is higher (but not double) than if only males were used. But, we now have the data to determine if males and females respond differently and, if not, a higher statistical power once the data are combined.

Also discussed above is that tissues are shared between multiple experiments. This sometimes extends beyond our lab, with us having, in the past, used brain tissue from rats intended for a cardiac experiment and others having come to collect peripheral tissues when we harvest brain.

If we embark on a non-standard experiment, pilot studies using small numbers of animals are always performed. We can thus determine whether the drug is tolerated or having the expected (or unexpected) effects before scaling up to full numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The choice of using rodents has many-fold advantages. Firstly, they are mammals and therefore share many of the features of humans (although consideration of their differences should be made). Secondly, they prove very amenable to genetic modification, making expression of genes associated with dementia more practicable than many other mammals. Thirdly, they are easily housed in animal units and do not require much specialised care beyond food, bedding and minimal enrichment within the cage.

Importantly, we have moved away from using transgenic mouse models of Alzheimer's disease, which have generally fallen out of favour with the research community as they overexpress amyloid precursor protein (APP), resulting in many off-target effects. Instead, we use APP knock-in mice which were recently developed that avoid this overexpression artefact and also ensures expression of the proteins at the right time and place.

A further advantage of moving away from the transgenic models that we have previous employed is that the incidence of seizures in the transgenics is relatively high. Our previous licence indicated that up to 25% of the so-called TASTPM transgenic mice die suddenly from a severe tonic-clonic seizure; a phenotype shared by several other models. Having moved to the more refined knock-in models, this phenotype is no longer common and occurs no more frequently than in the in-bred background strain (i.e. very rarely).

Our aim is to combine factors to exacerbate Alzheimer disease-related phenotypes in order to produce better models of disease and thereby further our understanding. To this end we will take the best available models available at the moment (discussed above), which currently are the NL-F mice from RIKEN (https://doi.org/10.1038/nn.3697) and cross these with mice that have a genetic risk factor for the disease, for example the R47H mutation of the microglial gene TREM2. Alone, this mutation causes little effect, at least at the level of welfare of the whole animal. However, when crossed with the APP knock-in mice we hypothesise that the disease symptoms will be exacerbated. On top of this genetic risk, we will incorporate an environmental risk for the disease, obesity, by feeding mice a high-fat diet. Our choice of diet is such that it represents a more typical Western diet than many high-fat rodent diets available – for example, many contain 60% fat, while we will use a 40% fat diet that is much closer to the content of, for example, a typical hamburger. As we are trying to model neurodegenerative disease, some of our mice will develop a harmful phenotype. However, upon the onset of clinical symptoms, animals will be killed humanely for use in experiments. Generally, however, most experiments are aimed at the stages leading up to neurodegeneration and therefore most animals will never reach this stage.

However, particularly with new models additional checks are taken to ensure that unexpectedly or unusually rapid development of potentially harmful phenotypes are not occurring.

Where surgeries are employed, every care will be made to minimise pain and distress. Anaesthetics will be used during the sterile procedures and analgesia provided peri- and post-operatively. Animals are then closely monitored during the initial stages of recovery until we are confident that recovery is proceeding appropriately and monitoring can be reduced.

Why can't you use animals that are less sentient?

We always use the animals of least sentience that is suitable for the question asked. However, in modelling a human disease of old-age that largely affects cognition, we cannot avoid using an appropriately sentient mammal for many of the experiments proposed here. Most of our experiments do not involve animals that have undergone surgery. Most are bred and then remain in their home cage until they are killed for tissue harvest, the only scientific procedure being that they are genetically modified (although this genetic modification can be harmful in that it causes an Alzheimer's disease-like phenotype).

If appropriate, we use organotypic slices that are prepared from 5-day-old pups, prior to much postnatal development. These cultures develop to mature neurones and retain the typical hippocampal trisynaptic connectivity and importantly are readily amenable to manipulation in vitro. However, their major limitation is that they are no longer in an intact animal and therefore influences of the rest of the brain and periphery are no longer present. However, on some occasions the initial effect of a manipulation and assessment

of dosage can be ascertained in an organotypic slice before being applied to a more relevant system. It also allows serial observation of effects over time without repeated intervention for an animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our protocols are designed to enable us to use the most appropriate drugs and methods. So, should new anaesthetics or analgesics become available and recommended by the NVS or NACWO, we can readily change to these.

We have recently increased the frequency of post-operative monitoring to ensure that recovery proceeds as expected.

Housing very aged mice is not typical for many colonies. In terms of monitoring for welfare purposes, mice are changed to a different protocol aimed at care of aged animals from the age of 15 months. This protocol provides frequent monitoring for weight loss and frailty, identifying fatty lumps versus tumour growth, deterioration of eyes and potential behavioural phenotypes associated with neurodegeneration. If any signed are detected, daily monitoring is instigated and declines in health causing undue suffering would mean that the animal is killed for experimentation immediately.

Our institutional standard is to provide some environmental enrichment. All home cages are supplied with nesting materials, a shelter, a cardboard tube and wooden chew toys. As discussed, single housing is avoided wherever possible so that mice have a social structure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have several hard copies of Wolfensohn & Lloyd, Handbook of Laboratory Animal Management and Welfare 3rd Ed, which we always direct new PIL holders to and also reference anything we would like further clarification of.

Current best practice according to the Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement, 2001 (https://doi.org/10.1258/0023677011911345) is referenced in this PPL and should always be referred to for procedures involving administration of substances.

The UFAW handbook on the care and management of laboratory and other research animals, 8th Ed edited by Robert Hubrecht and James Kirkwood, is also available as an e-resource via the Library to complement the above.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institutional Biological Services offer courses related to the 3Rs, alongside literature and email notifications of advances. When regulations or best practice changes, we readily adopt them to improve the welfare of our animals.

The bottom line is that for the welfare of our animals but also for the most reliable and translatable results of our experiments the aim is to have the healthiest and least stressed animals possible within the contstraints of the experiment.

30. The nervous system influences on pancreas development and physiology

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

pancreas, islet of Langerhans, diabetes, development, in vivo imaging

Animal types	Life stages
Zebra fish	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to assess the cellular and molecular pathways used by the central and peripheral nervous system to regulate pancreas development and pancreas function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Pancreatic islets of Langerhans are made up of different cell types, including the endocrine hormone producing cells, blood vessel cells, and nerve cells. Of the five endocrine cell types, the most well known are the insulin producing beta cells that lower blood glucose levels and glucagon producing alpha cells that raise blood glucose levels. Release of

these hormones from the pancreas needs to be tightly regulated, and changes in this balance could result in diabetes. While communication between different endocrine cell types have known roles in regulating pancreas function, the role of nerve cells has been debated. This is in part due to the limitations in our ability to study endocrine cell activity in living mammals upon changes in nerve control. The goal of this proposal is to use the zebrafish as a model to study the role of nerve cells in the development and function of the endocrine pancreas. Given their optical transparency and rapid development, the zebrafish can be used to visualize and track the developing nervous system in the same animal. Furthermore, we can dissect the impact of different nerve cells on the pancreas. This work is critical for understanding diabetes onset and progression given the changes in nerve networks are observed in patients with different types of diabetes.

What outputs do you think you will see at the end of this project?

This work is expected to provide new knowledge about the nerve signalling pathways that regulate pancreas development and function. The primary expected benefit is publications of new scientific knowledge about how specific nerve cell types improve pancreas development and function.

Who or what will benefit from these outputs, and how?

The data produced in this project will be presented at national and international conferences and published in academic journals. Additionally, the results of this research will be shared with the general public through outreach/public engagement activities, including the department's Diabetes Specific Patient and Public Involvement Group. The new information will allow for the better understanding of how nerve signalling regulates pancreas development and function, from how single cells behave to how they are organized to form an efficient organ.

Changes in the nerve network in the pancreas has been observed in post-mortem human pancreas samples from patients with diabetes. Yet, our knowledge on whether these changes are a cause or consequence of the disease is very limited. This project will provide important insights into the role of nerve cells in the development and function of the pancreas, which in the long-term could help our understanding of diabetes onset and progression and advance therapeutic developments for the treatment of diabetes.

How will you look to maximise the outputs of this work?

The findings will be made available through publication in open-access journals and presentation at scientific meetings. New genetically altered animals generated will be valuable and made available to other scientists interested in studying organ innervation and/or pancreas biology. The findings will also be shared with the general public through outreach/public engagement activities, including the department's Diabetes Specific Patient and Public Involvement Group.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 12500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There are several advantages to using zebrafish to study how the nerve cells and the pancreas communicate. (1) The eggs are fertilized externally, (2) the embryos develop quickly, and (3) the embryos and larval zebrafish are transparent. All together, these advantages allow us to execute detailed and non-invasive live animal imaging to study organ development and function. The metabolic and signalling pathways controlling the zebrafish physiology and cell behaviour are very similar to mammals, which makes the zebrafish a good model for studying human diseases.

Genetic engineering techniques can be used to make genetically altered zebrafish to carry out these studies. This includes inserting new genes not normally found in the zebrafish or introducing mutations that alter the expression or function of a gene of interest. By inserting new genes, we can make genetically altered zebrafish that can light up specific cell types or change cell behaviour by turning on the expression of specific proteins. To make these genetically altered animals we need to first inject the genetic material into zebrafish embryos, grow up a batch of ‘potential founders’ to adult stages, then screen for fish that have inserted the genetic material into the sperm/egg cells. Not all of the ‘potential founders’ will have the genetic material inserted in the sperm/egg cells and the efficiency of this process is dependent on several factors. Once a good ‘founder’ is identified, the genetically altered zebrafish will be maintained through mating the adult zebrafish and growing up their offspring, a process termed ‘rederivation’. Since zebrafish can give offspring up to 18 months of age, new generations of genetically altered lines will have to be started prior to 18 months.

These genetically altered adult zebrafish are critical for the outlined studies because they provide the offspring for our non-invasive live imaging in embryos and larvae. Prior to 120 hours post fertilization, the pancreas of the fish already has a structure that is more like adult mammalian pancreas and the cells in the pancreas can produce hormones (including insulin and glucagon) to help control blood glucose levels. Importantly, a rich nerve network is present in the pancreas and our data suggest that this nerve network is important for pancreas function.

Typically, what will be done to an animal used in your project?

We will be generating, breeding, and maintaining genetically altered zebrafish that are not expected to display adverse effects in adult stages (including weight loss, tumors, and abnormal feeding/swimming behaviour). These genetically altered animals will supply the offspring that will be used in the project studies.

The genetically altered animals will be housed in standard conditions and routine welfare assessments will be conducted.

What are the expected impacts and/or adverse effects for the animals during your project?

The adult genetically altered zebrafish are not expected to display any adverse effects during the project.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The highest severity that all animals could experience in this protocol is mild.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

One cannot study the dynamic role of nerve cells on pancreas development and function by simply culturing the cells in a petri dish. First active blood flow influences the development of the pancreas and second there are different types of nerve cells in the pancreas. It is difficult to recreate the exact network of nerve cells and blood vessels in a cell culture setting. We therefore need animals to understand what happens to the different cell types in the pancreas as we model the role of nerve cells.

Which non-animal alternatives did you consider for use in this project?

In vitro cell culture studies of pancreatic tissue obtained from human cadaveric donors with nerve cells could serve as a complimentary non-animal alternative for this project. Once we have narrowed down the specific pathways, we will assess the impact of selected proteins on cell behaviour using cultured pancreatic cells/tissue slices and pancreatic cell lines.

To advance scientific knowledge, computer modelling will be used to predict the changes in how pancreas cells communicate upon alterations in nerve activity.

Why were they not suitable?

In vitro cell culture studies will not fully re-create the complex network of different nerve cells in the pancreas and the active blood flow that exists in a live animal. In order to study how the nervous system influences organ development and function we need to first conduct studies in live animals where the structure and function of the nervous system and the pancreas are intact. The data generated from these studies are required for the generation of computer models and the design of specific in vitro cell culture studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our work using genetically altered (GA) fish lines for the past 6 years, we estimate that 12500 animals will be needed. This estimated number of animals is based on the number of genetically altered lines that will provide the offspring required to complete this project. All of the studies will be conducted on embryos prior to being capable of independent feeding, which will allow us to reduce the number of protected animals used. We will obtain established GA fish lines whenever possible.

Approximately 75% of the studies will use GA fish lines that are already established. During the course of this 5 year project, we plan to establish 20 new GA lines, based on our current number of preliminary targets and the number of inducible tools not yet established. From our experience, the efficiency of finding a good founder when generating new GA lines can range between 1 in 20 to 1 in 150 adults. For new GA lines, we will start by growing 50 fish for the first round of screening. More fish will be grown and screened if the initial screening does not produce a good founder. We estimate an average of 100 potential founders will be grown per line. All newly generated and imported GA lines will be frozen (with sperm collection form 4 different males per line).

50 GA lines will be housed at any given time to provide the offspring for all the studies conducted on embryos prior to being capable of independent feeding. Adult zebrafish can be maintained for up to 18 months of age; therefore, a maximum of 5 generations of rederivation over five years will be required to maintain the GA lines. Based on our experience, a maximum of 40 GA fish will be maintained per GA line. This will ensure that the animals are housed in a socially enriched environment and enough number of adults will be available to provide the offspring for the proposed studies. Although adult zebrafish can provide approx. 200 embryos per round of breeding, following selection of the transgenes and/or mutations of interest, the actual number of embryos we can use is lower

depending on the number of simultaneous readouts. For example, in an experiment requiring pre-selection of 6 GA combinations, 10 mating pairs will be required to provide enough offspring per experimental group. All unused offspring will be euthanized prior to being capable of independent feeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R's Experimental Design Assistant and the ARRIVE guidelines have been used to reduce the animal numbers. All of the studies will be conducted on embryos prior to being capable of independent feeding, which will allow us to reduce the number of protected animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use genetically altered animals that can provide multiple readouts (including cell identity and cell activity) and implement computer modelling at later stages once a robust dataset has been obtained.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically altered zebrafish lines that will provide the offspring required to complete this project. All of the studies will be conducted on animals prior to being capable of independent feeding; thus eliminating the requirement of conducting studies on protected adult animals that are expected to experience greater distress. The offspring from the genetically altered zebrafish lines will allow us to visualize the experimental readouts with non-invasive microscopic imaging.

Why can’t you use animals that are less sentient?

The adult genetically altered animals are required to provide the offspring for the experiments on the larval animals. It is not possible to conduct our studies in species that are less sentient, including fruit flies and worms, because they do not have a pancreas. However, all of the studies will be conducted on animals prior to being capable of independent feeding, where we would expect less suffering in comparison to studies on

adult animals. To study the nerve-pancreas cross-talk, we need to be able to control the activity of the nerve cells in live animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Routine welfare assessments on the animals will be conducted. Animals will be monitored for their growth pattern, swimming behaviour, and feeding behaviour. Genetically altered animals will be grown and maintained until they reach a maximum of 18 months of age. Fish exhibiting any unexpected harmful phenotypes will be killed, or in the case of individual fish of particular scientific interest, advice will be sought promptly from the named veterinary surgeon and assigned Home Office Inspector.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow published guidelines issued by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay informed by reading scientific articles and published guidelines by the NC3Rs, attending zebrafish and other research conferences, and liaising with the named veterinary surgeon, named animal care and welfare officer, named information officer, and named training and competency officer.

31. The pathogenesis of a neglected tropical skin disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Mice	adult, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Neglected tropical skin diseases (skin NTDs) are a group of skin infections caused by different types of organisms. These diseases, defined by the World Health Organisation, affect the world's poorest people and are an important factor in trapping communities in a cycle of poverty and disease due to their association with stigma, disability and mental health problems. The aim of this project is to better understand the processes that occur for one particular skin disease caused by a bacterial pathogen. By understanding the disease better, we can achieve our long-term goal of designing better treatments that might help the infections heal more quickly. At the end of this project we aim to have identified at least one drug, ideally already licenced for other diseases (so-called "drug repurposing"), that could be tested in the future.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Skin NTDs include diseases that can cause extremely serious skin ulcers that may grow to cover large areas of the body, such as an entire arm or leg. Affected individuals are usually found in remote farming communities in developing countries, where access to healthcare is difficult. Current treatments take several months, may require hospitalisation and often leaves patients with large wounds that need either skin grafts or even limb amputation. Better treatments that improve wound healing are urgently needed as this will reduce the burden on patients (reducing deformity and long-term disability) and their families (since many patients are young teenagers requiring treatment far from home). This project will contribute to a fuller understanding of disease development and allow better treatments to be found.

What outputs do you think you will see at the end of this project?

The main outputs of this project will be increased knowledge of how skin disease caused by this organism occurs. This is very important as relatively little is known about NTDs. Outputs will mainly be in the form of peer-reviewed scientific publications and conference proceedings.

Who or what will benefit from these outputs, and how?

In the short- and medium-term, the expected beneficiaries of this programme will be different parts of the academic community such as those working on the skin NTDs, and scientists interested in how specific genes are involved in disease formation. It may also be of benefit to those involved in drug development or the pharmaceutical industry as our findings could lead to the identification of new uses for existing drugs, or new drug targets for the disease.

In the long term, this project will benefit the populations living in regions where skin NTDs are found. This includes many countries of West and Central Africa, as well as other parts of South America and Australasia. These benefits are only likely to be seen some time after the project is complete.

How will you look to maximise the outputs of this work?

We will pursue an active publication strategy. New knowledge will be widely disseminated via publication in open access journal articles. Less impactful findings will also be published in Wellcome Open Research, a dedicated open access platform for results of this nature. We will frequently share our results with collaborators and results will also be disseminated at conferences, particularly the World Health Organisation's biennial meeting on skin NTDs.

Species and numbers of animals expected to be used

- Mice: 4300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use adult mice rather than neonates in this project because their skin is most similar to the human skin that we need to mimic. Mice are a well-understood model of bacterial skin infection for skin NTDs, and have been successfully used to develop treatments that are now given to patients. Inbred mice are also available, which helps by greatly reducing the number of variables that could influence the outcomes of our experiments making our results more reliable. Furthermore, mice can be genetically engineered to remove or alter the expression of genes that are also found in humans, therefore genetically modified mice can be used to study the role of these genes in the disease. The genetic models we will use have previously provided important insights into other medical conditions. Mice are the lowest sentient species we can use to achieve our scientific aims.

Typically, what will be done to an animal used in your project?

There are two types of experiments in this project, and each mimics a skin NTD by injecting material into the skin. The first type involves infecting the skin with a small amount of bacteria, by giving one injection either into the base of the foot or into the tail. These experiments last several weeks, up to a maximum of approximately 11 weeks, because the bacteria grow very slowly. The second type of experiment involves giving one injection of a purified bacteria-derived compound that is important for the development of disease, either into the tail or the ear. These experiments are shorter, lasting up to a maximum of approximately 2 weeks. These injections are given while the mouse is asleep, under a general anaesthetic.

In both cases, some of the animals might first be given a drug that causes the cells of a genetically modified mouse to change their DNA content. Alternatively, some of the animals might be given drugs that we think will change the ways the skin will respond to the injected material. The drugs will be given to the mice in a variety of different ways, and sometimes we might surgically implant a device that automatically delivers the drugs, if a vet thinks this will be less stressful to the mice overall.

We will carefully monitor the injection site and might sometimes use a medical device similar to an x- ray machine or an ultrasound, or other “imaging” device to see what is happening inside the skin. We will make sure the mice are asleep when we do this, so that it is less stressful to them.

At the end of the experiment, we will kill the animals at pre-determined points, while the animals are no more than moderately affected by the disease, in line with the severity limits of each protocol. We will kill them by a humane (Schedule 1) method. In some cases, we want to study whether the blood vessels are working properly, and these animals will be given a general anaesthetic first so we can inject a dye into their blood stream.

What are the expected impacts and/or adverse effects for the animals during your project?

In all Protocols we will carefully monitor changes in the general condition of the mice that might indicate that they are affected by the injected material. We will also look for potential side effects of anaesthetics, drugs given and/or the genetic modifications present. However, we are not expecting the mice to show any signs, like weight loss or indications that the animals are in pain, during any protocol. This is because the bacteria make a substance that is known to acts like a painkiller. We know that mice that have had material injected into one footpad may experience changes in the way they move about due to their foot swelling up. However, they will still be able to move around the cage without distress. We are expecting changes in the appearance of skin around the site of injection, but this is necessary to understand the disease. We will monitor all animals carefully to ensure that any harms are kept to a minimum and, if they occur, dealt with promptly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for the mice used in this licence is expected to be mild or moderate. Overall, considering all Protocols except the breeding of genetically modified mice, we expect 70% of the mice to experience a cumulative severity of moderate, with the remaining 30% of the mice experiencing a cumulative severity of mild. For the breeding of genetically modified mice, we expect 99% of the mice born on the Protocol to experience mild severity, with a low risk of one of the 10 genetically modified mouse lines we will breed showing moderate severity in 10% of the animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal use is needed to understand the disease in the context of a living being, and in particular live tissues with blood flow and an immune system. Two other important reasons are that the bacteria grow very slowly (much slower than the speed at which human cells divide in the laboratory) and the optimal growth temperatures are different (29-33°C, much lower than human body temperature of 37°C).

Moreover, revealing the role of individual genes and/or pathways requires the use of genetically modified mice in which these genes and/or pathways are changed so that we can study them. Finally, pre-clinical screening of compounds that might make good treatments is necessary in live animals before testing in humans can be considered. This is because a “fully body system” is needed in which the drugs are given in the context of a body’s metabolism as well as the immune system and blood circulation. None of these can be completely substituted by other techniques that don’t use live animals.

Which non-animal alternatives did you consider for use in this project?

This project involves a range of non-animal alternatives that run alongside animal use. For instance, we test cells isolated from donated excess human skin in laboratory assays, and examine skin biopsies from patients. This has allowed us to develop ideas about how the infections take hold. We also carry out basic biochemical assays on purified components of cells, in which we can investigate fundamental cellular processes. We have also considered developing a system in which a whole piece of donated skin can be maintained in the lab for several days/weeks.

Why were they not suitable?

The laboratory studies are informative, but tell us about cells bathed in nutrients in artificial conditions, rather than in their natural context. The clinical studies are informative, but present a snapshot at quite a late stage in disease which can mask the molecular "trigger" for the symptoms the patient experiences. The skin explant models do not survive for long enough for infections to establish, and also suffer from the temperature barrier, since they must be maintained at 37°C (not suitable for the bacteria causing this neglected tropical skin disease).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals used are estimated based on the experimental design within each protocol. For pilot studies we used a so-called Resource Equation to determine groups

sizes according to the law of diminishing returns. Data available from the scientific literature, or these pilot studies, are used to perform power calculations to determine appropriate group sizes for definitive experiments. We then estimated the total number of animals by adding up the number of animals used for pilot studies, the number of mice needs to perform studies that follow the evolution of the disease as it progresses, and the number used in definitive experiments. In each case this assumes a defined number of experimental endpoints, and that the experiment will be performed for 10 candidates over the course of the licence.

One Protocol describes the breeding of strains of genetically modified mice for use in other Protocols. We used calculators published in the scientific literature to estimate the minimum number of breeding pairs/trios would be required to obtain the required number of animals, and well-established general principles of animal husbandry to estimate the size of the colony needed to maintain this number of breeding pairs/trios for the minimum possible time to meet our scientific aims.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase, we took advantage of the NC3R's Experimental Design Assistant tool to consider all aspects of the experimental design. This included performing power calculations for group sizes, blinding and randomisation strategies, and statistical methods for the analysis of the data. This tool was extremely useful to ensure that the experiments will be performed in a way that reduces animal wastage by ensuring that the experimental data collected is robust and reproducible. Advice has also been sought and taken from a statistician on the design of all experiments in this license, and this will be an ongoing process throughout its lifetime.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wild-type in-bred animals will be supplied by registered breeder such as Charles River. Only the correct number of animals will be purchased. We will also breed genetically modified mice which will be used in the project. Where possible these will be bred as homozygous lines, and compared to wild type animals of the same strain supplied by registered breeder such as Charles River, in order to avoid wastage by breeding excess animals. We are aware of the issues around genetic drift, and will take this into consideration while maintaining any colony of genetically modified mice. Breeding of these animals is controlled by a breeding plan, devised by the NACWO, NVS and the research team, to minimise animal wastage. Wherever possible, we will re-use tissue that has already been collected in exploratory studies to investigate candidate pathways before embarking on definitive experiments.

Moreover, tissue will be shared within local and collaborating research groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use established models of a skin NTD in mice that involves injection of bacteria, or material derived from them, into the skin. This is necessary to mimic how the disease appears in patients. The injection sites are chosen based on established knowledge about how to model human skin diseases in mice. The footpad, tail and ear are all sites with characteristics most similar to human skin, and this means our results will be scientifically meaningful.

In both models we will perform pilot studies to compare within these injection sites to determine the approach which tells us what we need to find out about the disease, whilst causing the least distress to the animals. We will do this by taking measurements in the skin tissue after the mice have been killed, or by using imaging techniques on live mice, and compare them to our findings in patients. Like the infections in humans, these models are extremely unlikely to cause pain to the animals. Since the skin condition will deteriorate the longer the infections last, we have clearly defined humane endpoints to ensure that the animals never experience more than moderate severity. We will also use pilot studies to determine the earliest possible point in the experiment that answers our scientific questions so that these harms are minimised.

In order to test our ideas about the involvement of certain genes in the development of neglected tropical skin diseases, we will use two alternative approaches. These are either genetically modified mouse lines, or drug treatments known to reduce the activity of these genes. Since the genes we are interested in are also involved in other non-tropical diseases, they have been studied previously. This means we have been able to read these previous studies and select strains and/or drugs that cause the least harm to the animals.

Why can't you use animals that are less sentient?

Skin is a complex organ which has changed throughout evolution, and is very different between mammals (such as humans and mice) and lower vertebrates or invertebrates. Therefore, skin diseases in general require mammalian models. For skin NTDs in particular, some mammals (but not lower vertebrates or invertebrates) have been found to get similar diseases, especially when the infection is picked up from an environmental source that they are exposed to. In addition, mammalian skin changes before and after

birth, including changes to the way the blood flows and immune system works. This means that immature life stages are not appropriate for this project. The length of time taken for the infections to develop means that experiments under terminal anaesthesia cannot be used.

Therefore, an adult mammalian model is required to replicate clinical skin infections, and mice are the lowest sentience to meet the scientific aims and get results we can use to develop treatments for people in the longer term.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In order to minimise the possible harms, mice will be monitored with health checks that have been tailored specifically to them, and the protocol being followed. We will use anaesthesia and/or painkillers as advised by a vet to prevent and control any potential adverse effects (including stress or pain). We will implement additional care should specific animal husbandry be advised due to the models (for instance, the use of soft bedding/nesting material). We will perform pilot studies that will enable us to further refine doses, injection routes and the length of experiments so that we can use the most refined protocols. Where possible, we will preferentially use the model involving injected purified components, rather than infection with live bacteria, as these experiments are shorter. Our results will be reviewed regularly and integrated with new knowledge/experience/other publications.

In order to reduce the stress to animals, and hence the need for restraints, during the taking of animals' measurements we will previously undertake regular familiarisation handling of the animals and will use positive reinforcement. For instance, we aim to use refined handling techniques such as tunnel handling or cupping to reduce stress. We will also minimise the number of times each animal is handled by combining techniques where possible (such as measurements and drug administration if required on the same day), and using anaesthesia/sedation should this be advised by the NACWO/NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidelines from the NC3Rs and the Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. We will follow the PREPARE guidelines and checklist when planning our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The 3Rs are regularly discussed and best practice shared during the User Forum for the unit, which the research team attend and participate in regularly. Members of the team also sit on our institution’s AWERB. These animal welfare meetings, advice disseminated by the NACWO/NVS/NIO, and accessing regularly updated on-line sources of information

about the 3Rs (such as NC3R, Norecopa, Frame), will allow us to keep up to date with advances in the 3Rs and implement them in this project.

Throughout this PPL, we will review our results regularly and integrate any new knowledge/experience from other publications and collaborative network.

32. Understanding and treating preterm birth related pregnancy disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Pregnancy, Preterm labour, Ascending infection, Cervix, Neonatal infection

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand why preterm labour occurs, and how it can be treated or prevented.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ultimately we hope to be able to identify new treatments or tests for preterm labour.

Around 50,000 babies are born preterm in the UK each year – these babies are at increased risk of premature death and of disabilities in later life. This work will improve our understanding of why preterm labour occurs as well as developing and testing potential preventative treatments.

What outputs do you think you will see at the end of this project?

Our work aims to benefit women who are at risk of- or have had a preterm birth. Over 50,000 women and their babies experience preterm birth per year in the UK, with major cost to the NHS and to society as a whole. Preterm birth is associated with serious neonatal morbidity and mortality, particularly for those infants born before 32 weeks gestation. Preterm birth is the single largest direct cause of neonatal mortality in the United Kingdom and worldwide, being responsible for more than 1 million neonatal deaths globally. There are 54,000 cases of preterm birth per year in England and Wales, which accounts for approximately 8% of all live births and a quarter of these are associated with preterm deliveries before 32 weeks gestation.

Our goal is that our work will lead to clinical trials of novel agents and interventions for the prevention of preterm birth and the adverse neonatal effects of preterm labour, such as death and disability due to brain and other organ damage caused by infection/inflammation.

This programme of work is designed to establish detailed mechanistic pathways leading to interactions between the cervix in pregnancy and ascending infection which may contribute to premature delivery. Furthermore, it will enable identification of novel biomarkers with the potential for development of preventative therapies in women at high risk of preterm birth.

Within the next 5 years we hope to make significant scientific contributions to the field and within the next 5-10 years we hope to be able to translate some of these findings to clinical use. Given that our group has vast experience in running pregnancy-related clinical trials, we feel these are realistic timescales.

Who or what will benefit from these outputs, and how?

The main beneficiaries of the programme from scientists to policy makers, and the public are outlined below:

- The scientific community involved in preterm birth research: This research will benefit those academics who are carrying our similar or related work in the field of preterm birth and will bring us closer to full understanding the mechanisms behind premature birth.

- Scientists investigating mucosal immunity. It will also benefit those working in mucosal immunology.

- Scientists, clinicians in the field of public health investigating the consequences of preterm birth, and the potential lifelong impact on the child. Interest in defining preterm birth risk using biomarkers (including specific dominant vaginal microbiota communities) has expanded rapidly in the past few years. Furthermore, recent research has also focussed on the potential lifelong impacts of prematurity on the affected child. Our

research will help inform those working on large mother-child cohorts of novel associations worth investigating amongst women at high-risk of preterm birth.

• Translation to Clinical research: We have the potential to highlight mechanisms that may be manipulated by clinicians and pharmaceutical companies. Therefore, this work may lead to generation of biomarkers or drugs for medicinal purposes to prevent premature birth.

• Health professionals and policy makers responsible for the care of women who deliver their babies preterm and their children. It is our ultimate aim that this research will have a lifelong benefit for the health of pregnant women and their children. We already have established several channels of dissemination to health care providers and policy makers, and expect that the proposed programme of work will inform Policy and public health practice within the lifetime of the PPL.

• The lay community, especially pregnant women and their children. As a Tommy's Baby Charity Research Centre, we communicate closely with Charity's press officers, in order to disseminate research findings to the lay public.

How will you look to maximise the outputs of this work?

We hope that our animal studies will contribute to the development of a novel diagnostic test for preterm labour with the next 5-10 years. Even if we are unsuccessful in this aim, we will add to the sum of knowledge on the mechanistic pathways of preterm parturition. Our department has an excellent track record in publishing our research, and in training the next generation of researchers; so that the academic community and individual students will also benefit from this work. We plan to present our results from our animal studies through both publications and presentations at local and international conferences to share the knowledge we gain with others in the medical and scientific community.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the minimal species required to address our research questions, and its biology is sufficiently similar to the human for it to give us helpful information. We monitor all animals carefully throughout the entire duration of the experiments. Where more refined approaches become available during the studies, we will adopt them.

We will use the following mouse strains:

Conventional strains (CD-1, BALB-c/, C57BL/6, C57BL/6N-Tyrc-Brd)

Mucin homozygous knock out mice (Muc5ac-/-, Muc5b-/-) and heterozygous mice (Muc5ac+/-, Muc5b+/-)

The above knock out mice will be used so that we can manipulate the cervical mucus plug which is central to understanding preterm birth mechanisms in humans.

Adult male and female mice will be used for breeding in Protocol 1.

Non-pregnant (controls) and pregnant adult female mice will be used in Protocol 2. Pups born to these dams will also be used for imaging purposes under this protocol.

Neonatal pups and then post-weaned adult male and female mice will be used in Protocol 3.

Typically, what will be done to an animal used in your project?

These studies involve trying to understand and prevent preterm labour. The majority of animals will have an induction of preterm labour (for example, by injecting inflammatory or anti-progesterone substances). We will also test preventative therapies for the prevention of preterm birth and then monitor for improvements in outcomes in the mice and offspring as a result of preventing preterm labour.

Typically, mice bred in protocol 1 will be used in studies defined in Protocol 2. Following identification of genetic status, animals may be:

a) maintained for conventional breeding (this protocol)

b) used in other procedures in this Project which require the genetic modification or supplied to other Projects with authority to use genetically modified animals of this type

The majority of these mice will then be used in Protocol 2. Non-pregnant mice or time-mated pregnant mice will be used, and they will be housed together for companionship. Animals will be treated with therapeutic agents (or control) aimed to modulate labour/preterm labour. Administration will occur prior to preterm birth induction or at the time of preterm birth induction. Preterm birth induction will be induced from embryonic day 14 to embryonic day 17. The majority of our animals will have preterm birth induction either via ultrasound-guided intrauterine injection or intravaginal administration and will

therefore not undergo any surgery. A range of preterm birth inducing agents will be used including LPS toxin and live microbes. The doses of LPS and bacteria which we administer have already been determined to have subclinical effects and, therefore, we do not expect to see any adverse effects. Mice exhibiting clinical symptoms may show immobility, piloerection and fever. If the duration of this exceeds 24 hours, they will be killed by Schedule 1 method. All cases of preterm birth induction will occur under general anaesthetic using isoflurane anaesthetic. A small number of mice that may undergo

laparotomy will only undergo this single surgery and any other treatment will involve injection of agents to modulate parturition. We anticipate that 50% of our animals will undergo imaging, and less than 20% of these will undergo repeated imaging. At the end of protocol 2, all adult female non-pregnant and pregnant mice will be killed by schedule 1 methods or terminal exsanguination. Some of their pups may be kept alive and used in Protocol 3.

The pups will undergo behavioural tests described in protocol 3 to assess the effects of preterm birth on the brain and if the phenotype has been rescued by the preventative therapies.

Pre-weaned animals will undergo no more than 1 testing session of each step, no more than once per day, and lasting no more than 30 minutes per session. Adult mice will only be tested once and not for more than 1 hour per day. All animals will be killed at the end of this protocol by schedule 1 methods or terminal exsanguination.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocol 1:

Breeding will occur in this protocol so we expect to produce pregnant dams (protocol 2), non-pregnant females (protocol 2) and more animals to continue breeding in this protocol.

Breeding pairs will be kept for a maximum of 12 months after birth after which they will be killed by a Schedule 1 method.

Protocol 2:

In order to achieve our aims, we will stimulate preterm labour in some mice. Hence the adverse effects for the mothers and the pups include prematurity, which can be fatal. Some procedures used will result in transient pain (e.g. at injection site), followed with close monitoring. The majority of our experiments are of mild severity. The adverse effects of infectious/pro-inflammatory agents are sometimes of moderate severity; however these effects will be minimised at all times (see below). We will also assess preventative therapies in this protocol and will anticipate rescuing the phenotype and reducing any adverse effects related to preterm birth.

The preterm labour-inducing biological agent dose used will be the minimum necessary to achieve this phenotype as calculated by pilot dose studies.

For studies where preterm labour itself is under investigation, animals normally undergo preterm labour within 48 hrs and are normally culled no later than 72 hours after labour. Signs of inflammation include fever >38 degrees Celsius, piloerection, refusing to eat and a reluctance to move unless prompted. Any animal with these signs will be monitored closely (4x/day). We will monitor temperature with a rectal thermometer. If these signs do

not improve within 24 hours (ie. temperature <38 degrees Celsius, animal freely moving in the cage) the animal will be killed by schedule 1 methods.

For general anaesthesia, appropriate depth of anaesthesia often depends on strain. Therefore, under- or over-anaesthesia will be minimised/avoided by monitoring breathing rate (50-70 breaths/minute is expected). Adult mice will only receive inhalation anaesthesia, isoflurane. Hypothermia is a common adverse effect of general anaesthesia for surgery in rodents. Therefore, we will perform all surgical procedures on a thermally-regulated pad or in a thermally-regulated chamber (not exceeding 30 degrees Celsius). After anaesthesia mice will be observed until they are awake, in a heated recovery box before being returned to their cage.

For surgery, the animal will be monitored continually until it has fully recovered from the general anaesthetic. Possible pain during/after the surgery will be minimized using appropriate analgesia. Where ongoing signs of distress are observed (lack of mobility, hunching, piloerection, discomfort on handling) animals will be administered analgesia until they recover. If the signs of distress continue, despite analgesia, on the day of the procedure, the animals will be culled at the end of that day or within 6 hours of the procedure (whichever is sooner).

Complications including infection and wound dehiscence may rarely occur as a result of surgical manipulations. This will be minimized by the appropriate use of aseptic technique. Frequent observation (3x/day) will be undertaken following the procedure to ensure that wound healing and recovery is free from bleeding or infection. The animals will also be weighed on the day of the procedure and daily for the next 7 days. In the unlikely event of wound breakdown, a single attempt at closure of a healthy wound will be attempted up to 24 hours after surgery. Any evidence of bleeding during surgery will necessitate general compression until bleeding is stopped. In the rare event of bleeding from the wound or signs of infection (redness, swelling, pus exudate and discomfort over wound area) animals will be culled under Schedule 1.

We expect no adverse effects from preventative therapies tested as these compounds will be administered at sufficiently-low published doses as to have no clinical symptoms. However, any newly developed therapeutic compound may cause potential adverse effects including contact dermatitis or weight loss, which will be monitored for on a daily basis.

Protocol 3:

In this protocol, the majority of behavioural tests are not expected to cause any adverse effects. None of these observations or manipulations will cause pain and analgesia will not be necessary. During the Morris Water maze test the water temperature will be kept to an average temperature of 22 degrees Celsius (water will be testing continuously with a thermometer placed in the water). Mice will be placed in a thermally-regulated chamber (not exceeding 30 degrees Celsius) following each trial. If the animal does not swim when

placed in the maze or appears distressed, they will be removed from the trial and placed in the chamber to recover. They will not undergo any further testing.

If an If an animal falls during the cliff aversion test it is unlikely to cause any physical harm but the shock of falling may invalidate the behavioural test. We will monitor all animals closely and ensure there is padded tissue below the platform. If the pup does fall, we will perform no further trials in this animal.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

We aim to use 2500 animals over 5 years;

- 1500 at mild severity
- 1000 at moderate severity

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The whole process of designing and testing a therapy is as follows: i) It is tested in vitro, usually in more than one mouse line and, where possible, in cells differentiated from human induced pluripotent stem cells. ii) The therapies are finally tested for efficacy and, where appropriate safety, in the relevant model. The disease, preterm birth, we aim to cure is a multisystem disease, which cannot be modelled in vitro, neither in single cell cultures nor in mixed culture or organoid systems. Animals are ONLY used when human work is not possible, and when in vitro cell work is unsuitable, both because of the nature of the question being asked. To translate these technologies to the clinic, preclinical studies in mouse models are a minimum requirement. Mice in particular have proved to be a useful model for our studies of parturition due to similarities in the inflammatory and immune response to infectious agents. Usually, well-performed mouse studies, combined with toxicity studies in rabbits, outsourced to a CRO, are sufficient and higher animal studies are now no-longer a requirement for progression to the clinic for preventative therapies.

Which non-animal alternatives did you consider for use in this project?

We considered single cell culture, mixed cell culture or organoid systems.

Why were they not suitable?

The disease, preterm birth, we aim to cure is a multisystem disease, which cannot be modelled in vitro, neither in single cell cultures nor in mixed culture or organoid systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated numbers based on previous experiments and pilot data (see below).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments are designed using the NC3R's experimental design assistant and will be reported in line with ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For the primary aim of this project – preclinical experiments in a translational pathway, it is crucial to ensure experiments are sufficiently powered otherwise they may require repeating in their entirety. Our approach is to perform an initial sample size analysis based on data previously obtained in our laboratory. Where experiments are completely new, we will perform pilot studies with 4-8 animals per treatment group and use this data for statistical analysis in power calculations to determine appropriate sample sizes. For the majority of experiments, we will aim to have a significance level of 5% and a power of 80-95%. Many of our therapy interventions are emphatically therapeutic therefore this experiment tells us if we are on the right track. Most importantly, we can analyse numerous disease parameters. Therefore, we are able to select one of the least corrected parameters on which to base our power calculations for an additional fully powered study, if necessary. Where possible, we share material with our collaborators, thus pooling resources and minimising experimental repeats.

In vitro work is used where possible, either to avoid animal work all together, or to determine suitable doses, time periods etc. before embarking on in vivo animal work.

Using biosensors and bioluminescent bacteria we can specifically reduce the number of animals used in biomedical research, by enabling the monitoring of signalling pathways non-invasively, continually, rather than sacrificing cohorts of animals at repeated time points or, for example, performing repeated blood collections.

For previous multi-treatment comparisons, we have used one or two-way analysis of variance with an appropriate post-hoc test. For time series we used analysis of variance with repeated measures. All preclinical studies are performed fully randomised and fully blinded. Where possible, all end-point analyses are performed at the same time point. For our biosensor work, each animal provides its own baseline, and therefore serves as its own control. We can, therefore, normalise each animal against this baseline, minimising experimental variation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is our chosen animal model, for which there are usually appropriate genetic models. Lower organisms do not recapitulate the pathology of these diseases. Larger animal models are rarely required by the regulators for clinical translation, as mentioned above. Often, we work with models with an acute phenotype (e.g. infection-related preterm birth), with only a short period of time before onset of symptoms and requirement for euthanasia. Therefore, animals are not in discomfort for prolonged periods of time. Our team monitors our mice on a daily basis and, in some cases, more than once a day. They have accumulated many years' experience in recognising clinical symptoms of distress and pain.

Why can't you use animals that are less sentient?

Our main animal model is the mouse for which there are usually appropriate genetic models. Lower organisms do not recapitulate the pathology of these diseases. Terminally anaesthetised animals will not provide the disease phenotype acquired to study disease mechanisms and test preventative therapies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Any variation from the norm is followed up with careful weight monitoring and more prolonged observation. Almost invariably, when distress and pain are recognised, the animal is euthanised, since, in our experience, sick mice tend not to get better – they are prey species and so will hide their symptoms until they are very unwell. Also, we recognise that the longer a sick mouse is maintained, the poorer will be the quality of the data, so it is better to euthanise early and justify the early time point, than euthanise late, causing the animal suffering, just for the sake of hitting a time point for which the data will likely be skewed. In our most recent bioimaging work, we demonstrated that it was possible to image young mice noninvasively, freely moving, within the confines of a dark chamber. The animal can even be provided with wet chow for the duration of the imaging. This dispenses with the need for anaesthesia and there is no disturbance of the animal by the operator. The imaging environment, other than being novel, is, therefore, similar to that of their home cage during the dark cycle. Pups are marked, at birth, with a subcutaneous foot tattoo if necessary. This allows us to randomise and blind treatments in each litter and still be able to identify each animal for its lifetime with no need for ear clipping.

We do not anticipate that any experiments will be of “severe” severity.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

ARRIVE guidelines 2.0

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

On the NC3R website and by attending regular webinars provided there.

33. Gamma delta t cells and body surface immunity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, immunosurveillance, inflammation, T cells, epithelium

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to describe the biology of intraepithelial lymphocytes (IEL) which comprise a large compartment of immune cells sitting at body surfaces, such as the skin, gut or reproductive tract. IEL are particularly enriched in evolutionarily conserved T cells known as gamma delta cells, which can play important roles in key physiologic processes such as dietary adaptation, and in protecting against infections, inflammation and cancer. However they are very poorly understood. To redress this, we aim to establish how specific sets of gamma delta T cells become associated with particular tissues during their development; how they are retained; which forms of local disorder they can respond to; and the biological outcomes of their actions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Body surfaces are the most susceptible anatomical sites to infection, cancer, and inflammation that collectively make up some of the most common and demanding clinical burdens. They can also be sites for resection and transplantation. By understanding how local gamma delta T cells protect their local environments from various types of disorder or disruption, we can add to our fundamental immunological knowledge while potentially improving clinical diagnostics and therapeutic strategies. Indeed, our previous work provided the foundation for two biotech companies committed to applying gamma delta T cell biology to cancer and inflammatory disease with first-in-human trials commencing in autumn 2021.

What outputs do you think you will see at the end of this project?

The following outputs can be anticipated:

• Peer-reviewed primary research publications. To place this in context, our research led to the publication of 25 peer-reviewed primary research papers in the 4.5 years covered by our current license.

• Patents. Our work has led to novel discoveries which have been protected as intellectual property by our host institutions, so that they may be commercialised for practical clinical application(s).

• Abstracts. Our work is primarily undertaken by post-doctoral researchers and PhD students who are encouraged to attend international meetings to which they submit abstracts for presentation and discussion.

• PhD theses. Our laboratory is currently training 4 PhD students whose written, published theses can be anticipated to contain work undertaken as part of this project.

• Public engagement via research updates on open-access websites, etc.

Who or what will benefit from these outputs, and how?

We consider the following impacts:

• Short-term: publications describing our results may have immediate impacts on: [i] other research teams, influencing their own experimental plans, studies, and interpretations; [ii] post-doctoral trainees whose career progressions can be largely shaped by the outcomes of the research; [iii] in rare cases, the impact may be to influence current public health policy, as was recently achieved in the context of COVID-19.

• Short-to-medium term: awarding of PhD degrees to those undertaking studies described in this licence application.

• Short-to-medium term: the establishment of UK and international collaborations to expand upon our findings, as manifest in our successful application to the MRC and its Indian counterpart (DBT) for Anglo-Asian COVID-19 studies.

• Medium-to-longer-term: impacts on textbook immunological knowledge.

• Medium-to-longer-term: the results, including patents filed, may accelerate the development of novel diagnostic and therapeutic modalities for infection, cancer and inflammation.

• Longer-term: changes in clinical practise.

How will you look to maximise the outputs of this work?

We shall disseminate our findings via the outputs described above: papers, abstracts, theses. We shall disseminate our findings via conference presentations.

Where appropriate, we shall disseminate the implications of our findings by public engagement. We shall upload our data and provide lay narratives on open-access web-portals, etc.

We receive advice from experts in science translation about which of our findings are appropriate for filing patents, thereby maximising opportunities for clinical development of our findings.

Species and numbers of animals expected to be used

- Mice: 77,275 split over 20 protocols over 5 years

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the ideal organism for our research for five reasons

First, it is a long-standing choice of immunologists via which they have successfully identified many of the key operating principles of the immune system, such as the connectedness of innate and adaptive immunity, and made advances upon which major clinical interventions are based, e.g. the successful introduction of cytokine blockade for treating rheumatoid arthritis.

Second, there is an immense spectrum of reagents available permitting the thorough, incisive, and comprehensive obtainment of reliable information from the experiments undertaken.

Third, there is an extensive and evolving bibliography of protocols that inform the design of experiments, promoting reproducibility, reducing excessive need for pilot studies, and enhancing the reduction and refinement of animal use.

Fourth, reflecting extensive breeding programmes and the ease with which it may be genetically engineered, the mouse has emerged as an extraordinarily powerful mechanistic system, permitting the unequivocal assignment of function to particular genes and molecules. It is important to recognise here that there will be occasions when our parallel studies in humans implicate a specific gene or pathway in, for example, regulating cancer immunosurveillance. The power of mouse genetics permits this implication to be tested irrefutably by assessing cancer progression in mice rendered mutant for that gene/pathway. This means that the mice may experience higher severity of discomfort when compared to wild-type mice challenged with tumour cells or carcinogens, and hence will be appropriately monitored (see below).

Fifth, many protocols have been developed and are being refined for highly effective, reproducible longitudinal monitoring.

We examine all developmental stages of this organism, because our research seeks to understand the developmental association of T cells with tissues which often occurs in the fetal and neonatal periods; the cells' responses and functions in adults; and the long-term consequences, e.g. the durability of immunological memory in older individuals.

Typically, what will be done to an animal used in your project?

The main foci of our research are cancer, infection, inflammation and transplantation.

Many animals used will not be treated in any way, but will at different ages be culled solely as sources of different cells and tissues for study in the laboratory; hence they will experience no ill effects.

For experimental models, mice will be injected with and/or otherwise exposed to cells; and/or to agents such as chemical irritants, toxins, ultraviolet light or other mutagens of the kind that humans are commonly exposed; and/or to microbes or viruses, so as to induce cancer or infection and/or inflammation. They may also be injected with and/or otherwise exposed to cells and/or agents, such a chemical inhibitors or biological substances such as immune cell stimulants known as cytokines, that are known or hypothesised to ameliorate or to enhance susceptibility to cancer or infection or inflammation or transplant rejection. In both cases, cells/agents will be administered by the most suitable route leading to very minor effects on the animals.

In some cases, fragments of organs, specifically skin, will be explanted into a recipient mouse, so that the immune response to that may be assessed.

The number of procedures we use will be kept to the minimum needed to answer our research questions with the highest levels of certainty. At any signs of undue distress in

the experimental mice, the experiment will be terminated, and the course-of-events recorded to inform future practice.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the mice will experience no ill effects. Most animals in our breeding and monitoring programme will experience little and/or only transient adverse effects. It is possible that the inter- breeding of novel genetically altered strains will be accompanied by unanticipated adverse effects, such as we observed over a brief period for a very small percentage of mice deficient in butyrophilin- like 1 (Btnl1) which developed hydrocephaly. Our intensive monitoring of our animals means that any adverse events are detected quickly; animals culled; and alternative breeding strategies and/or experimental methods (e.g. bone marrow transplantation of cells from one genotype to another) are adopted.

Different challenge models can be anticipated to provoke different outcomes depending on the age and the immune status of the host. Following some challenges, particularly in young mice and in potentially immunodeficient strains, a standard quantitative measure of the host response is weight loss (more correctly termed weight retardation although this retardation is only temporary, after which the mice develop at the same rate as normal). Some other potential signs of distress may include hunched posture, lack of appetite and ruffled fur. Nevertheless, challenged animals will be monitored closely during periods of potential distress to ensure that appropriate action is promptly taken should any develop severe clinical symptoms.

Mice exposed to cancer cells and/or carcinogenic agents, and those carrying genetic mutations predisposing to cancer may develop cancers for which we have approved protocols for tracking and measurement. The time to develop cancer can vary considerably depending on the nature of the challenge/mutations and the genetic background of the mouse, particularly in relation to its immunocompetence.

When human cells are used for adoptive transfer, the animals may develop human cancers, phenotypically similar to the human disease. The time to develop cancer will again vary depending on the primary tumour cells engrafted and whether or not other cells, e.g. human immune cells, are co- administered. In our experience, the mice are not overtly affected by the cancer growth. It is expected that the mice will have a mild systemic effect.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most mice on our breeding and monitoring protocols that experience any discomfort at all will experience mild severity. As mentioned above, inter-breeding of novel mutant strains

can be accompanied by unanticipated adverse effects. In such cases, breeding and monitoring will be classified as moderate severity.

All the mice on our experimental protocols will fall under the categories of mild/moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of our studies, as considered above, is to understand how body surface gamma delta T cells in vivo offer local protection from infection, cancer, and inflammation, and how they regulate the response to organ transplantation. Clearly, some substantial component of our work needs to be undertaken in vivo, although we quickly move to studies in vitro to investigate the underlying biochemical and cell biological processes that underpin events in vivo (see below). We examine immune responses in vivo in humans, but those studies are limited by three key factors: [i] tissue-resident T cells are our main focus of interest and yet it is challenging to monitor immune phenotypes in tissues as opposed to blood;

[ii] it is extremely unusual to find humans whose genotypes allow unequivocal causative association of a gene and/or biochemical pathway with a biomedical outcome / phenotype; [iii] it is appropriately impossible to challenge humans with a spectrum of infectious agents, potential carcinogens, and inflammatory substances. To overcome some of these issues, we are using human (and mouse) organoids much more than before, and trying to overcome a substantial limitation on their current use, namely the appropriate anatomical intercalation of tissue-intrinsic immune cells, particularly gamma delta T cells, with their host tissue organoids. Importantly, our track record shows that as soon as we make key findings in animal model systems, we set up experiments using human material to test their implications for human health and disease.

Which non-animal alternatives did you consider for use in this project?

As is evident from our publication record, we have where appropriate undertaken human studies (e.g. immune-profiling) and mathematical models and shall continue to do so. Likewise, we have established experimental protocols that employ human and mouse cell lines and organoids that reduce the need for investigations in vivo.

Why were they not suitable?

As considered above, the irrefutable power and value of non-animal alternatives falls far short of recapturing the spatial and temporal dynamics of the interactions between tissue-intrinsic immune cells and their host tissues both at steady-state and after those tissues have been challenged by clinically relevant agents, including microbes, carcinogens, inflammatory substances, and tissue grafts.

Nonetheless, knowledge gained from non-animal alternative systems has helped us refine the design of our animal experiments, enhancing reproducibility and thereby reducing the numbers of animals included in our studies, and limiting the scale of severity.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use based on two parameters:

1) The number of mice we have used in these protocols over the last five years; note that this number has evolved as our knowledge has grown and the incisiveness of experimental protocols has improved (see Appendix C).

2) The projected number of mice that we estimate we shall use on different protocols in the next five years based on our current research focus and the progression of the field in the global scientific community.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have undertaken many steps in this regard.

1. We have and shall continue to investigate the ready availability of genetically-altered strains from the community, prior to generating any de novo. If new lines need to be generated, we shall employ the most efficient techniques such as Crispr-Cas, which reduce animal numbers by virtue of the fact that targeted animals can be generated within fewer generations, particularly when we wish to examine the impact of a genetic change on different mouse background strains.

2. We have been extremely scrupulous in our choice of experimental systems that have wide dynamic ranges that permit us in single experiments to measure immunological improvements and deficiencies, respectively, and that have low likelihood of batch effect variation, that we monitor with programmes such as Tableau. As part of good laboratory

practice, protocols for each experiment defining the objectives, experimental procedures, intended effects, and endpoints will be circulated to all those involved in the care of the mice.

3. We select challenge agents based on: [i] ready availability in defined forms that permit high experimental reproducibility in standard control strains; [ii] our own experience of the challenges and/or ready availability of robust protocols within the community; [iii] our relevant scientific knowledge, e.g. that roles for the agent in gamma delta T cell biology have not already been discounted based on rigorous experimentation; [v] pharmacogenetics, e.g. the probability that an agent may yield important biological data based on clinical observations made in the realms of human genetics and treatment responses, respectively.

4. In our choice of challenge agents and experimental protocols, we consider that all the competences and reagents required for optimal downstream outcomes analysis exist, thereby maximising the amount of information to be obtained from single experiments. As an example of our choice of a tumour challenge model, we ask if so-called antigen-MHC tetramer reagents are available to detect tumour-specific alpha beta T cell responses that may be regulated by gamma delta T cells.

5. In every instance that the experimental design permits, we shall use a non-animal alternative (human immunophenotyping; organoids; immune cell - tumour cell co-cultures in vitro, etc).

6. All models used will be assessed such that we shall employ the minimum severity of disease (e.g. tumour burden; inflammation; infection) required to show an effect.

7. We are not wedded to a single approach. For example, in asking what information skin gamma delta IEL receive at steady state from Skint proteins, one could use mice in which Skint1 can be inducibly deleted. But, we chose instead to plan experiments in which we block the Skint1- gamma delta IEL interaction by intradermally administering anti-Skint1 antibody to ears of WT mice. This reduces animal use because it does not require a complex breeding programme for the mutant strain, and because (for example) a contralateral ear injected with control immunoglobulin can be used as a control for anti-Skint in a single animal, rather than requiring a second, control mouse in which gene deletion was not induced.

8. Where pathophysiologic responses to challenges are to be described, e.g. immune surveillance of cancer; capacity to mount immunity to infection; acceptance of graft, power calculations will be undertaken to determine the minimum numbers of mice required to demonstrate a phenotypic effect. Ideally, we use power of >95% and P=0.01 as thresholds, since our experience shows that conventionally-used thresholds are often too lax for many experiments, and may result in the longer run in more mice being used in attempts to resolve the consequent experimental uncertainties. Reaching stringent standards is promoted by well-established experimental systems with high signal-to-noise

across broad dynamic ranges (above); nonetheless, to limit animal group sizes it will on many occasions be satisfactory to apply thresholds of >80%. Typically, t-tests or chi-squared tests (or non-parametric equivalents) will be suitable for respectively comparing time to phenotype onset, disease burden, or quantitative immunophenotype between groups, but given the multiplicity of measurements and group comparisons we shall also frequently employ ANOVA. According to the number of groups compared, corrections may be required, e.g. Benjamini Hochberg, to reduce false discovery rates.

An informative power calculation for a typical experiment is as follows: suppose WT mice have 25% less tumours than genetically engineered mice deficient specifically in interferon gamma production by gamma delta T cells, n=40 vs n=30, sd=8, then to reveal a significant difference between the groups requires 23 mice in each group.

9. In many cases, the numbers of animals required will be reduced by longitudinal measurement of responses, by serial blood analysis and/or by optimised and ever-evolving intravital imaging protocols. Hence, the immune response to a challenge may be measured weekly in a set of six mice over a period of six weeks, rather than requiring six mice to be sacrificed weekly across that period. Such longitudinal usage provides essential information on the development or not of immunological memory, but does require approval for successive sequential administration of agents and blood sampling.

10. Unnecessary variation in animal cohorts will be excluded by use of gender and age-matched controls housed under identical conditions, as we have published, and likewise transgenic, knockout, and “knock-in” mice will routinely be generated or obtained on the same genetic background, and, where relevant, not subject to experimentation until the appropriate number of backcrosses has been completed.

11. Guidance in experimental design can be consulted at:
https://eda.nc3rs.org.uk/experimental-design

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Apart from good experimental design, we shall use the following measures to optimise the number of animals used in our project:

Pilot studies using limited number of mice to determine feasibility of new experiments
Detailed consultation with others who have used the relevant protocols

Where possible, tissue will be shared between other members of our laboratory so that we optimise information gained from the single unit of analysis – the individual mouse.

Mice will be bred efficiently; i.e.: only enough breeding pairs will be set up to meet the requirement of ongoing experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Models and Methods:

We shall mainly use mouse models of:

[i] tumours (subcutaneous, intradermal and metastatic tumour models).

[ii] inflammation (administration of agents by gavage, intranasally, intravaginally, or epicutaneously) to induce inflammation of the relevant body surfaces.

[iii] infection, via gavage, intranasal, intravaginal, or epicutaneous exposure with systemic exposures as means to test, for example, the impact of local protection on systemic immunity.

[iv] vaccines and adjuvants via gavage, intranasal, intravaginal, or epicutaneous exposure with systemic exposures as means to test, for example, the impact of local protection on systemic immunity.

[v] wound healing.

[vi] skin-graft transplantation.

Suffering and discomfort:

Using past experience, the literature, consultation with colleagues locally and globally, all mouse models that we plan to use will be assessed such that we shall employ the minimum discomfort severity burdens required to show effects. For example, in B16 melanoma tumour challenges, we primarily employ the B16 F0 subline for skin tumours because it is demonstrably less aggressive than the F10 subline that is widely used by others. Moreover we undertake pilot dose response studies before adopting final SOPs.

Because the value of our data will be enhanced by comparison with similar studies by the community, mice will be killed at stages of disease-progression consistent with those widely used throughout the UK and international scientific laboratories.

Most animals produced under the breeding protocols are not expected to exhibit any adverse phenotypes and most immunodeficient mice are also unlikely to show adverse symptoms because of the high health status of the animal facilities. However, it is not

possible to fully predict the nature or severity of any potential mutation: mutations in gamma delta T cell functions critical for gastrointestinal cancer immuno-surveillance may impact upon steady-state barrier functions, for example, water retention. With these risks anticipated, newly developed or obtained strains will be closely monitored and animals exhibiting any unexpected harmful phenotypes will be killed by schedule 1, or in the case of particular scientific interest, advice will be sought, first from the NVS, and if appropriate, then from the local Home Office Inspector.

Invasive mouse procedures required are minimal. Analgesics will be used as required.

Why can’t you use animals that are less sentient?

Gamma delta T cells emerged in and have been conserved in vertebrates, so there is no capacity to employ nematode or fly genetics in their study. While some immune studies make use of fish, they are inappropriate for our studies because of the paucity of reagents, methods, and supporting literature, and because the contexts that we study would be at an even greater physiologic distance from clinical settings.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We shall refine the procedures we are using by increased monitoring according to clearly articulated guidelines, e.g. FELASA “Working Group on Pain and distress”; EC “Endorsed Severity Assessment”; and NCRI. Thus guided, we shall determine the earliest endpoint possible on an experiment-by-experiment basis to allow a valid scientific outcome to be achieved. Our approaches to this, e.g. consultation with colleagues; reference to the literature; our own experience, are described above.

Assessment will be made of pain and distress, as measured by normal and provoked behaviour; movement; physical signs such as altered respiration rate; animal posture (huddling or hunching) skin and coat changes such as piloerection or overgrooming; inactivity; body weight; inflammation of injection sites; and comments on the animal’s general appearance.

Where procedures seem likely to be distressing to animals (e.g. the highly accurate measurement of subcutaneous tumour burdens using calipers), mice may be anaesthetized although this will be avoided if accurate measurements can be obtained without anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As mentioned above, we will follow best practice guidance published by:

FELASA “Working Group on Pain and distress”

EC “Endorsed Severity Assessment”

NCRI guidelines

National Centre for the Replacement, Refinement and Reduction of Animals in Research guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will stay informed about the advances in the 3Rs by regularly staying updated with the guidelines specified in the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs): (https://www.nc3rs.org.uk).

I shall regularly appraise my staff of any changes to the guidelines and ensure we try, at every juncture to implement these advances effectively.

34. Developing anti-malarial transmission blocking interventions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph

Key words

Malaria, Plasmodium, Transmission, Mosquito, Vaccine

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to discover and develop new ways to treat or prevent malaria transmission and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The project aims to contribute to the control and elimination of malaria. With 3.3 billion people at risk of infection and 229 million people infected with malaria every year, resulting in over 409,000 annual deaths (WHO World Malaria report 2020). The development of novel anti-malaria blocking strategies is a major technological drive, specifically requested by funders and policy makers, to complement the status of currently utilised anti-malarial interventions.

By focussing on the manner in which the Plasmodium parasite (the causative agent of malaria) passes between mosquitoes and humans, this project aims to find new vaccine candidates or drug targets within the sexual stages of the parasitic lifecycle. Targeting the parasite in this way will arrest the completion of the Plasmodium lifecycle by preventing transmission, thus preventing disease. In addition, the project includes the study of the fundamental biology of the malaria parasite, performed to advance our general understanding of this globally important disease.

What outputs do you think you will see at the end of this project?

Based on our key collaborations with pharmaceutical companies, understanding of the parasitic biology as well as previous experience, working under multiple projects we are confident we will be able to achieve success within these broad aims of the proposal. Specifically, aim to:

• Identified ~15 several specific parasite targets for further drug or vaccine development;

• Explore the ability of these targets to block the transmission of a rodent malaria parasite.

• Validate modes of action by modifying targets in transgenic parasites to specifically test these ideas.

• Identified multiple (approximately 5) of the most efficacious candidates to translate to human studies in subsequent work.

Further outputs will include; multiple peer-reviewed publications and pre-prints, presentation at national and international conferences, and public engagement activities.

Who or what will benefit from these outputs, and how?

The work proposed to be performed under this project licence aims to contribute to the control and possible elimination of malaria. With billions of people at risk of infection, causing substantial disease and death annually, the disease also presents a significant barrier to socioeconomic development in Sub-Saharan Africa. As such, the development of new anti-malaria blocking strategies is a major objective. Our group has crucial ongoing links with non-governmental organisations, clinicians and field sites, and our research is used to inform the use of current and future anti-malarial interventions.

Specifically, the benefits of our research within the next five years will be to identify new targets which could be used to generate anti-malarial therapeutics. The results of this work

will be disseminated via presentation at conferences and in scientific journals to aid others in the field of malaria research.

In the short term, the benefits of this work will be limited to the identification of key molecules that control how malaria parasites transmission to the mosquito host, and the identification of anti-malarial interventions. In the medium to longer term, the development of novel anti-malarials in a clinical settings is the desired goal.

How will you look to maximise the outputs of this work?

1) Dissemination of results by publication (of both positive and negative data).

2) Presentation at national and international scientific meetings.

3) Collaboration with a range of malaria/vaccine/drug related scientific groups.

4) Links with specialist field-based labs, funders, clinical sites, and non-governmental organisations, to aid the translation of basic scientific research to a therapeutic product.

5) Multiple outreach/public engagement activities.

Species and numbers of animals expected to be used

- Mice: 11500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The rodent malaria parasite Plasmodium berghei (P. berghei) is one of few available safe (non-human infectious), versatile, biologically relevant and reliable species to study transmission of malaria from vertebrate to insect host and back again. The adult laboratory mouse is the most convenient and widely accepted host for rearing P. berghei.

Typically, what will be done to an animal used in your project?

Typically, the majority of animals within this project will receive an injection of phenylhydrazine into the abdomen, with subsequent infection with Plasmodium berghei 3 days later. At the dosages used, phenylhydrazine induces the production of cells called reticulocytes, which Plasmodium berghei preferentially invades. Infection will occur by injection into the abdomen or blood vessel.

In a number of experiments within this project, animals will be humanely killed following a mosquito feed to infect mosquitoes. In these cases, the mice maybe previously immunised (by injection into the abdomen, blood vessel, muscle, or under the skin), or drug treated

(by administration of experimental anti-malarial compounds by oral treatment, or by injection into the abdomen, blood vessel, or muscle) prior to parasitic infection to determine the transmission blocking efficacy of novel anti-malarial agents. Throughout the work within this project, an animal will only undergo one procedure.

Rather than allowing the mouse to wake up following a mosquito feed, some mice will be killed whilst under deep anaesthesia from which they are not allowed to recover. An unconscious/anaesthetised mouse will feel nothing while we remove a large blood sample.

A number of mice described within this project will not be infected with Plasmodium berghei but will be instead immunised to produce antibodies against proteins derived from the malaria parasite. Typically, these mice will undergo immunisation by injection into the abdomen, blood vessel, muscle, or under the skin. This may be repeated up to a maximum of 3 times per mouse. The resulting immune response will be monitored by examining the amount of antibody present in small volumes of blood taken from mice. When the antibody level is sufficient, these mice will be given anaesthetic and killed by removal of a large blood sample before the animal wakes up. Antibodies will then be purified from the blood and used in the lab.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts/adverse effects throughout the project can be split into four different classifications:

1). Impact of infection with Plasmodium berghei:

Infection of mice with P. berghei can result in development of cerebral malaria or anaemia in under 1% of cases. This results in clear signs of distress, which include pallor, bristling of hair, reduced mobility, hunched posture, lethargy/weakness, weight loss and respiratory disease.

2) Impact following blood sampling:

Adverse effects of blood sampling are very rare and can be short lived (under 1 hour). Localised bruising may occur in this time period.

3) Impact of immunisation:

When immunising mice, transient pain at injection site may occur. Animals will be monitored following immunisation and any animals showing signs of prolonged distress will be killed, though no adverse effects are envisaged from straight DNA or protein/peptide vaccinations. Observations include regular weight monitoring and daily observations looking for signs of ill health. Expected adverse effects at the site of immunisation include granulomatous reactions, swelling and redness with or without loss of hair over the injection site, and rarely ulceration. These will occur in <10% of animals.

4) Impact of treatment with anti-malarials:

Adverse effects resulting from treatment with antimalarials are not expected. Animals will however, be monitored for general signs of deviation from normal health.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice:
- Mild severity: 17%
- Moderate severity: 83%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Human malaria can be caused by several species of Plasmodium. However, due to practical, ethical and safety restrictions, transmission stages of malaria are almost impossible to use when working with lethal human parasites, such as Plasmodium falciparum. Therefore, the use of animals, such as mice, are currently the only way to study the full malaria transmission cycle of malaria.

The rodent malaria parasite Plasmodium berghei is one of few available safe, relevant and reliable species to study the transmission of malaria and generate genetically altered parasites. Plasmodium berghei cannot be grown in culture, offering only limited insights into the biology of the parasite. As such, the full replacement of the mouse model is not currently possible. Additionally, there is currently no alternative to animal species that can be used to test with regards to vaccine or drug sensitivity studies in a biologically relevant environment.

Which non-animal alternatives did you consider for use in this project?

Use of cultured human malaria parasites (i.e. – P. falciparum or P. vivax) was considered, as was the potential use of enhanced 3D cell culture systems.

The use of non-antibody systems (e.g. lab produced agents that bind to molecules) to target proteins essential for malarial transmission was also considered.

Why were they not suitable?

Unfortunately, despite these options, the need to continue experiments with P. berghei in mice remains necessary. Transmission stages of malaria are unsafe and challenging when working with lethal human parasites, therefore the use of animals remains the only way to replicate a full malaria transmission cycle. The ability to perform this is essential for the examination of transmission blocking interventions (both vaccines and drugs). In addition, the generation of transgenic parasites in other malarial species is at an efficiency far less that that observed in the P. berghei model.

There is no organid or culture system that facilitates the development or maintenance of the transmissible stages of P. berghei.

The use of non-antibody systems to target transmission-related molecules does not offer characteristics that are suitable for target identification (e.g. clearance rate, binding affinity). Their use would also not result in information regarding the immunogenicity of vaccine candidates.

We additionally continue to explore viable alternatives to replace the experimental procedures requested, by the use of new experimental culture systems on human malaria parasites.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals proposed to be used under this project licence will be reduced by using well- established, robust systems for parasite propagation, transgenesis and mosquito infection. The robustness of these systems greatly reduces the number animals used, as nearly 100% of animals become successfully infected upon inoculation. Our long experience (over 15 years) of working with these systems enables us to reduce the number of animals used by careful planning of experiments, using appropriate controls and replicas to avoid unnecessary experimental repetition. Tissue sharing will be performed where appropriate.

Typically, blood feeding on live animals is required for mosquito colony maintenance. This has been requested in previous project licences, however, we have successfully adapted our mosquito colonies to feeding via an artificial membrane, greatly reducing the number of rodents requested within this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For statistical/experimental design, in the majority of occasions, direct experimental measurements are not made on animals during the course of the procedure; instead, animals are used to generate parasite material for subsequent analysis. In these cases, the minimum number of animals needed to produce this material is used based on the acceptable minimum standards for publication of the results.

If statistical analysis is needed, the numbers of samples/experiments/observations is determined by previous experience, hundreds of available literature and using the most appropriate statistics. In all cases, all experiments are designed to minimize the number of animals used, e.g. data from initial experiments is examined, and only if justifiable the full experiment is carried out (e.g. duplicate and triplicate). For assessment of transmission in the mosquito, specialist statistical analysis is typically required due to non-normal distribution of Plasmodium transmission. All analysis will be 'performed taking into consideration variation between individual animals and the distribution of the parasite in the mosquito. We will additionally utilise in-house enhanced statistical methods to assess parasite distribution/transmission in mosquitoes, ensuring appropriate design of experiments.

In addition to more specialist analysis, the NC3Rs' Experimental Design Assistant has been utilised, along with PREPARE guidelines, and the field accepted-mimima for statistical significance

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In all cases, all experiments are designed to minimize the number of animals used, e.g. data from initial pilot experiments is examined, and only if justifiable the full experiment is carried out (e.g. duplicate and triplicate). For assessment of transmission, mosquitoes will be examined for intensity of infection and prevalence. Wherever possible, tissue-sharing of animals will be performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use rodents infected with Plasmodium berghei to examine malarial transmission to mosquitoes.

In almost all our work, the infecting parasite dose is large (to ensure rapid establishment of infection), animals are monitored daily and are used within a few days of infection. The use of phenylhydrazine enables rapid establishment of the required parasite levels before clinical signs. Under these conditions, infections are well tolerated, causing little discomfort to mice with animals typically displaying a normal behaviour. In procedures outlined here, mice are humanely killed prior to the start of chronic infection, reducing the severity of any procedures. During generation of transgenic parasites, drug treatment follows well-documented regimens and is known not to induce adverse phenomena.

During antibody production, resulting immune responses are monitored by taking very small blood samples by the least invasive method. Different immunization programs are established to raise robust immune responses, ensuring maximum chance of success using the smallest number of animals under minimal duress.

For the study of potential anti-malarial drugs, animals will be given doses based on data derived from lab-based studies, and initial doses used are not anticipated to cause health problems since compounds have been tested for toxicity. Known anti-malarials, which are investigated for their effects on transmission or the parasite in the liver, will be used at doses equivalent to those already routinely used for humans and are unlikely to result in toxicity effects.

Why can’t you use animals that are less sentient?

Less-sentient animals cannot support a malaria infection.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

To improve the quality of life of the animals we will:

- Reduce contingent harm by using group housing where possible and not using the single housing of mice. This in turn reduces any stress and stereotypical behaviour.
- Use environmental enrichment (EE), within what is available to us at our animal facility. In general, EE is an animal housing technique composed of increased space, physical activity, and social interactions, which in turn increases sensory, cognitive, motor, and social stimulation. Igloos, running wheels, saucer wheels, tube mazes, and other objects in the housing environment promote exploration and interaction. EE can be maintained through restraining (e.g. tube maze whilst handling), thus minimizing stress when for example an injection is needed.
- Keep animal transportation to a minimum.

- Use scoring sheets to monitor the health of animals undergoing procedures. v). Utilise non-tail handling methods.
- For any methods using anaesthesia, mice will be monitored closely for the duration of their recovery.
- If required, following veterinary advice, palatable pain medication will be used to reduce discomfort following the formation of local inflammation post vaccination.
- If more than 50 bites are given, mice are treated with saline solutions (under the skin) to compensate for blood loss.
- Eye drops administered to prevent dry eyes while the animal has no blink reflex while under anaesthesia.
- Heating pads to ensure maintenance of body temperature.
- A specialised Malaria Rodent Endpoint score sheet to help with the monitoring of our animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use guidelines from the Laboratory Animal Science Association (LASA) to make sure all experiments are conducted appropriately. In particular we will follow the information described in 'Avoiding Mortality in Animal Research and Testing'.

To ensure refined experimental design we will follow the PREPARE guidelines for planning experiments, and for thorough, responsible reporting of results we will follow the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We constantly review methods for replacement, reduction and refinement provided by the scientific literature and our support team within the facility, attending workshops to stay up to date.

During this project we will continue to be informed about the 3Rs by regularly checking our institute's 3Rs search page, and being registered fo regular NC3Rs emails and newsletter updates. Regular reference to guidance documentation provided by the Laboratory Animal Science Association (LASA) and the RSPCA will be made.

We will also ensure continued contact with the organisational teams in the facilities where our animal work will be conducted. Any new recommendations will be incorporated into our experimental plans wherever possible.

35. The chicken chorioallantoic membrane (cam) assay for biomaterial testing in tissue engineering

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Skeletal tissue engineering, Stem cells, Bone, Angiogenesis, Biomaterials

Animal types	Life stages
Chicken (Gallus gallus domesticus)	Embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to evaluate biomaterial structures and growth factors developed for the repair of the skeleton using a live, pre-clinical chick egg chorioallantoic (blood vessel) membrane model. Our rationale is centered on the urgent need to develop tissue regenerating biomaterials capable of activating and growing both blood vessels and bone to help patients repair their damaged and diseased skeletal tissue.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With the advent of an ageing population, skeletal tissue loss due to injury or disease is steadily growing. This significantly impacts the quality of life for the patient over time and

has socio-economic costs for healthcare providers. For example, each year in the UK there are over 50,000 primary hip replacement operations at a cost in excess of £350million. Hence, for reconstructive bone surgery and fracture repair, the need to develop better techniques and alternative bone therapies is vitally important.

The combined development of blood vessels and skeletal tissue are critical in the growth and maintenance of our skeleton and more importantly in the repair of our damaged bones. Without the cell communication from these two sets of tissues we find that our skeleton fails to function properly and is therefore less capable of successfully repairing itself.

Currently, our knowledge of the processes involved in cross-talk between these two tissues is incomplete. If we can understand the scientific unknowns of the developmental interaction of these cells and factors from these two sets of tissues, then we can develop and design smart biomaterials to mimic these interactions to successfully regenerate skeletal tissue lost due to trauma or disease.

The embryonic chick egg membrane model, which has a rapid growth of blood vessels (21 days), is ideal for understanding the role of the blood vessels in skeletal tissue formation. This rapid blood vessel growth can help provide information in how it is involved in tissue and organ development.

Conversely, this model can also be used to study diseases by simple exposure to stimulatory factors, hormones or drugs, or by transplantation of tissues, isolated cells or materials. This model has been used for many years in the cancer field studying the role of blood vessels in the development of tumours. Recently, we have used this model to try and understand the cellular interactions of the blood vessels in growing skeletal tissue. We have discovered that chick-derived blood vessels grow into and provide vital stimulatory factors and nutrients to the cells attached to or inserted into bioengineered products to help fix bones. As a result, this living model has become an important tool for testing experimental bioengineered materials to optimise the best combined design which can simultaneously promote the development of blood vessels and skeletal tissue.

A better understanding of how bones repair themselves could help develop treatments for patients and animals suffering from diseases and fractures affecting the skeleton. These include non-healing bone fractures and weakened bones due to diseases like osteoporosis and bone cancers.

What outputs do you think you will see at the end of this project?

The chick egg membrane model is being used in these studies to gain new insights that will help us in the development of tissue regenerating biomaterials to rebuild broken bones.

Specifically;

1. we aim to develop suitable new formulations of materials that can stimulate bone and blood vessel formation individually and simultaneously, to generate products that can be used in the clinic.

2. we will present our findings at scientific and medical conferences and we will publish these outcomes in peer-reviewed scientific journals.

3. we aim to use the latest technological applications to find out how the biomaterials stimulate the bone and blood vessel cells/tissue to grow and repair, and to use these imaging devices to track tissue development to reduce the numbers of embryos required to study these new biomaterials.

Who or what will benefit from these outputs, and how?

As with all our research goals the aim is to develop improved therapeutic strategies and products for patients who suffer from skeletal diseases and bone trauma injuries. We envisage that these project protocols and novel biomaterials have the potential to provide new treatments to help rebuild bones and skeletal tissues. In addition, the findings from musculoskeletal research can be applied to the veterinary field due to similarities in orthopaedic conditions between people and animals, leading to novel applications in a 'one health' approach.

Beneficiaries will include:

1. Patients and animals suffering from broken bones and skeletal diseases. Healthcare providers.

2. UK, EU and worldwide tissue engineering/biotechnology companies involved in tissue regeneration, stem cell biology or developing innovative tissue scaffold technologies,

3. the academic community in the generation of new protocols and avenues for skeletal tissue regenerative research.

Many materials we use in our studies are biocompatible and currently used in clinical practice for other applications. Modifying them to enhance their properties can result in new therapies in a relatively short time frame, benefitting patients due to the known track record of efficacy and safety. However, with the complex materials proposed the final outcomes for patients may require months to years of investigation due to thorough testing to ensure safety and efficacy prior to use in the clinic.

When these new treatments become available, we envisage growing the tissue constructs in the laboratory and transferring the regenerated samples to the patient in theatre. Ultimately, we believe this work will be translated to the clinic and benefit patients within the National Health Service and the wider medical community in the area of musculoskeletal repair. Alternatively, the development of materials that exploit the

regenerative potential of the patient's own repairing cells would minimise costs and hasten the material's therapeutic implementation.

In addition, the information from this project, including the study protocols and techniques will be made freely available via publication in peer-reviewed journals, in order to benefit patients, other researchers, doctors, vets, and pharmaceutical companies who are involved in the development and assessment of novel therapeutics which target the repair and regeneration of skeletal tissues due to disease and injury.

How will you look to maximise the outputs of this work?

Training & Collaborations

The team undertaking the research will acquire skills and expertise in the chick egg membrane model, blood vessel and skeletal stem cell biology, biomaterial development, imaging techniques in real-time such as 3D x-ray scanning and 3D optical imaging. Training will be achieved through an extensive local network of multidisciplinary collaborations within the University and established national and international collaborations in regenerative medicine.

In addition, the model and research work will be communicated to our Student & Postdoctoral researchers to provide new students and established researchers information regarding the models available for research projects in the University and to foster collaborative projects. We are part of a UK wide collaborative group network where we share our methods and results from acellular materials that will be available to be tested in skeletal and other tissue/organ regenerative models.

Education & Public engagement

The information and findings generated from this project will be presented through the scientific community by presentations at national and international meetings. We will also communicate findings to the public through outreach activities such as the University and laboratory open days for GCSE and A-level students in tissue regenerative medicine.

Progress of the study and results will be regularly presented at ongoing teaching events and public lectures. The press office of our university will also publicise the results.

Species and numbers of animals expected to be used

- Domestic fowl: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The development of bone into a large anatomical tissue requires a fully functioning blood supply. Unfortunately, this cannot currently be created in the laboratory; therefore, an animal model is needed to provide the missing functional blood supply to test our bone development and regenerative biomaterials.

From fertilisation of the egg to hatching is 21 days, as a result, the blood vessel membrane surrounding the chick embryo develops rapidly. The advantage of the model is that any tissue placed upon the membrane will become surrounded and nourished by blood vessels and continue to grow. This model is unlike other models that use mice or rats where anaesthesia and a surgical procedure on the animal has to be performed to implant the test biomaterial to investigate its tissue regenerative properties. With the chick membrane model, no invasive surgery is required, we are just "piggy-backing" the chicks external blood supply to grow our skeletal bioengineered scaffolds. In addition, as the chick embryo has a subfunctional immune system, biomaterials incorporating cells from any species can be placed onto the membrane, the limit being the overall size of the explants (probably not in excess of 2-3cm3).

Typically, what will be done to an animal used in your project?

A typical experiment will comprise of the egg (day 3) having a light shone upon it (candled) to determine fertilization and that the embryo is growing. Under sterile conditions a window (approx. 1-3cm2) is created in the eggshell and our test skeletal bioengineered materials are placed on the highly rich blood vessel membrane surrounding the chick embryo. The window is sealed over with clear film and the chick is placed in a 37-39oC humidified (45-60%) incubator to continue its growth and development. Only one procedure per egg is done, which is the opening of the eggshell window and the placement of the sample(s) on the membrane. The duration of the experiment is no more than 16 days. Gestation of the egg is 21 days.

In addition, another method is applied whereby the contents of the egg at Day 3 is carefully separated from the eggshell and placed in a sterile dish. Thereafter, a bioengineered material is placed onto the membrane covered and returned to the incubator to grow and develop up till day 19. At varying time points in both models imaging of the developing embryo, blood vessel membrane and biomaterial will be done either by microscopy or x-ray scanning.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects associated with this model are expected to be very minimal as we are not undertaking any harmful techniques on the developing embryo. There is a small risk of infection, however, this is minimised as all work is carried out in a sterile clean cabinet with sterile instruments and biomaterials.

When opening the egg shell window, there is a small risk that egg shell fragments will spill into the membrane, triggering a minor reaction. This actually helps the embryo as it normally uses the eggshell as a source of calcium to help grow.

There is also the potential to damage the blood vessels when creating the window as the membrane is close to the surface which can result in the embryo not developing.

With the shelless external membrane model, there is the potential to damage the embryo, blood vessel membrane and the yolk sac when transferring to a dish. This can reduce the development of the embryo.

X-ray scanning. There is the risk that radiation doses during a micro-CT X-ray examination may produce adverse effects such as delayed growth. However, doses of x-rays and duration of scans are way below any threshold that will cause problems to the animal.

Biomaterials. The adverse effects of the implantation of biomaterials are expected to be extremely rare. We will have tested the safety of these materials in the laboratory using cells, toxic and biochemical testing prior to consideration of implantation on the chick blood vessel membrane assay.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities will be gauged to be sub threshold to mild.

We will have tested the biomaterials prior to implantation in cell culture work to ensure there is no release of any toxic factors or toxicity to cells directly in contact with the material. As with all batches of eggs we receive, there is a very small percentage 2-3% that are not fertilised or do not develop normally to hatching during the incubation period. We anticipate that 95% of animals will have severity scores of subthreshold, and up to 5% will have a severity score of mild.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The development of bone requires a fully functional blood supply. Unfortunately, to date, this cannot be created in the laboratory with current tissue and cell culturing experiments, therefore an animal model is needed to provide a functional blood vessel network in order test the capabilities of new bone regenerative materials.

Which non-animal alternatives did you consider for use in this project?

Using laboratory cell culture experiments we are gaining as much information as possible to understand the functions and toxicities of new biomaterials being developed. In addition, we are studying the interaction of cells that make up the blood vessel and bone and how they combine to create 3-dimensional vascularised bone tissue known as organoids.

Artificial Intelligence (AI) modelling is now being developed to test biomaterials implanted into the chick membrane, so that this technique can be used to predict and determine how a new material will act in producing blood vessels and new skeletal tissue. However, there are limitations at the moment as this AI modelling is based on data provided from real studies. Once enough data has been accumulated for AI, this can, in principle, remove the need for the chick membrane model when researching new biomaterials in the future.

Why were they not suitable?

We have been unable to identify a cell model or a lab-based tissue/organ model that can copy a functioning blood vessel network essential for skeletal growth and repair. The generation of new bone requires multiple steps and the interplay of many different bone cells and blood cells in a co-ordinated manner in a 3-dimensional environment.

This interaction cannot be consistently or correctly reproduced under tissue culture conditions to meet the criteria expected to inform a clinical translation. In addition, in vitro model systems make it difficult to detect unexpected toxicities and observe the integration and degradation of materials over time.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any.

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used our current and past experimental data to inform us of an estimation of the number of animals required per group and the number of control and test groups to give statistically valid experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the design phase we have used chick eggs to reduce intra-group variability of biomaterials and by using this model and the results obtained has allowed us to reduce progressive experiments in mice models. We have designed experiments using the fewest animals consistent with obtaining statistically valid results as determined from our power calculations. Using the NC3R's Experimental Design

Assistant we have calculated the minimum number of eggs required for a determined amount of new bone and blood vessel formation in comparison to control implanted samples.

In certain cases, mathematical modelling may be used to predict the release of growth factors from biomaterials, allowing us to determine how many chick eggs we can exclude while still achieving a meaningful outcome in the experiments. In addition, multiple scanning and imaging of the chick membrane at different time points reduces the number of chick eggs required in these type of studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Rigorous laboratory testing will be performed on the newly constructed biomaterials before any candidates will be put forward for the chick membrane experiments.

Multiple analysis will be undertaken on biomaterial samples implanted on the chick membrane, this will include X-ray and microscope imaging, blood vessel scoring, biochemical and molecular analysis, and histology. The multiple data that can be derived from one sample will optimise and minimise the number of eggs to be used in a single study. In addition, real time optical/microscope imaging will significantly reduce the number of time points required to assess blood vessel development within the implanted biomaterial. This will further reduce the number of eggs required for one study involving multiple time point assessments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Refinement is any measure to reduce suffering, or improve welfare, throughout an animal's life; however, this is not always achievable depending on the research question.

The chick membrane model is especially valuable, given that it has the ability to reduce the number of conscious, sentient animals (mice & rats for example) in subsequent experiments and therefore limiting pain and suffering of other animals used in subsequent confirmatory experimental models. As already alluded to, the chick membrane provides a vascular supply distant from the embryo, which does not involve a surgical procedure or anaesthesia compared to other models investigating bone and blood vessel formation, such as the mouse subcutaneous implant model. Through laboratory testing and biocompatibility cell studies we expect the biomaterials implanted onto the chick membrane is not harmful to the chick embryo. However, we cannot be 100% certain when a biomaterial is implanted on the chick membrane that the embryo will not experience any harm from the implant integrating with the surrounding tissue. From previous studies we ascertain there may be a 5% chance that the embryo will experience a very mild level of harm.

The advantages of the use of chicken egg embryos for research are the reduced conscious characteristics whereby it is reported that their nervous system is underdeveloped until day 17. It is important to note that while procedures on the chick membrane are often perceived as harmless there are still mitigating risks such as damage to the membrane or factors released from the biomaterial which may be incompatible with the development of the embryo. To date, research that identifies the timing of pain perception in the chick embryos remains limited. This research understanding is essential for safeguarding the necessary welfare standards when using this experimental model.

Why can’t you use animals that are less sentient?

The chick embryo is the least sentient species that has a functioning blood vessel system that enables us to investigate the skeletal regenerative properties of biomaterials designed in the laboratory. This model allows the screening of many biomaterials to inform us of the most effective materials that can be applied to a more complex animal model. In the mainstay, this has the potential to reduce experimental testing in more sentient animals. Zebrafish models for testing biomaterials involve injecting or implanting materials into the zebrafish to test the inflammatory response, but it is known that fish have the capacity for nociception which may be greater than that of the chick membrane and it is also difficult to quantify vascularisation in zebrafish.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Standard operating procedures for the chick membrane models will be constantly updated in line with advances in new methods developed. Annual risk assessments are carried out on the model and close record keeping of the experiments and eggs helps us to determine problems that have arisen. This will allow us to develop new practises to further minimise

any potential harm to the embryo. On a technical note the incubators will be cleaned and regularly tested to be in correct working order prior to each study and throughout the duration of the studies. The temperature and humidity at egg level are monitored digitally and the incubator functions monitored by a thermometer. These digital readings are recorded daily and the egg rotation is ensured to be occurring daily as well.

The eggs are candled to ensure the chicks are developing correctly and this is repeated on the day prior to creating the eggshell opening. Any undeveloped or cracked eggs will be discarded.

Users of the model will hold a personal license, attained accredited training in the avian egg modules and will be trained and confirmed to be competent in all necessary procedures by experienced users of this model.

Careful implantation of materials with no/minimal disruption of the chick membrane will be performed so that the embryo is not directly contacted during the procedure.

Chick embryos will be humanely killed using a schedule 1 method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are a number of detailed publications on the chick membrane model that provide excellent guidance in the methodologies, study design and best practise that we will follow and adapt to our scientific research.

We will follow the ARRIVE guidelines which provide a 20-part checklist of the minimum information required to be reported by groups using animals in research. ARRIVE guidelines are essential to help overcome issues in science such as reproducibility, reducing bias and the correct use of statistical methods of analysis.

In addition we will follow and consult the Norecopa (Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with animal experiments) database platform and PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for better science experiments using animals to ensure that we are using the best defined models for our work in investigation of biomaterials for skeletal biology.

Importantly, the chick membrane model offers additional details that can be used to direct progression to preclinical animal models. In vivo trials, without a doubt, serve as a vital link between laboratory bench discoveries and clinical use of medicinal instruments, pharmaceuticals, and products. As a prior ‘screening' model, the chick membrane assay may theoretically minimise the weak association observed between laboratory experimental results and those from animal models. The chick membrane model, according to current evidence and our viewpoint, offers a rapid, cost-effective, flexible,

stable, and repeatable method for assessing materials prior to evaluation in animal models comprising of complex bone repair.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will consult the NC3Rs website to identify any changes that will be relevant to the workings of this model. In addition, we will follow the latest findings from the Laboratory Animal Science Association (LASA) and the PREPARE guidelines from Norecopa in better planning for research involving animals to prepare for better science and advance the 3Rs. Any changes will be implemented directly through the experimental design, and if necessary, through a project licence amendment. In addition, we follow the latest publications on using the chick membrane model and identify any new methods that reduce, replace or refine the chick membrane model workings. If applicable to this model in improving the 3R's we will request amendments from the Home Office to adjust the techniques/methods required and training or notification of relevant staff in updated techniques.

36. Development of novel therapy strategies for skin diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

genetic skin disease, gene therapy, skin sheet graft, topical delivery, inflammatory skin disease

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop specific and effective treatments for rare genetic skin diseases and common inflammatory skin diseases such as eczema.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In human beings, skin as the first barrier plays a protective role against infection and water loss and maintaining our normal bodily function. In patients who have inherited faulty

genes, the protective function of the skin is impaired or defective. These patients are vulnerable to infection, dehydration, and other conditions secondary to impaired skin. For example, in patients with a faulty gene called COL7A1, their skin is very fragile and often forms blisters following even minor mechanical stress and this symptom starts from birth. Moreover, in severe forms, chronic erosions with secondary infections can progress to widespread, mutilating scars and joint contractures, and aggressive skin cancers. All this can cause significant disability and affect the quality of patients' lives and sometimes is life threatening. There are no curative treatments for these genetic skin conditions and the development of novel therapy is, therefore, urgent.

Apart from rare genetic skin diseases, we are also concerned about the treatment for eczema, which is a chronic and relapsing inflammatory skin disease affecting 15-20% of children in the UK. Patients with eczema have redness, itching, flaking and blistering skin. In a considerable proportion of patients, eczema affects not only on the quality of life of the affected individual but also places a social, emotional, and financial burden on families. In these patients powerful oral immunosuppressive therapy is often required for many years with the attendant side effects and risks of long-term immunosuppression. Development of effective treatments for dermatitis (AD) are therefore highly desirable.

What outputs do you think you will see at the end of this project?

The skin diseases we have been investigating are debilitating skin conditions, causing significant illness, disability and severely affecting the quality of the patients' life. Currently, there are no curative treatments for these rare genetic skin conditions (less than 1 in 50,000 to 200,000 affected individuals) and no effective therapy for the common skin disease eczema.

This project is designed to develop new therapies for these diseases. If successful, our results will lead to treatment of these diseases to reduce patients' suffering and their families' burden. In addition, the outcomes of the research are likely to provide new insight into how these skin diseases contribute to skin disease, for examples, morphological changes, protein expressions, enzyme activities and lipid composition in the skin. We will present and publish these information and findings in national and international conferences and peer reviewed journals.

Who or what will benefit from these outputs, and how?

If successful, these therapies will provide clinical benefit to patients with rare genetic skin diseases or Eczema.

If the outcomes of our research are promising, we will transfer the research from 'laboratory bench' to 'clinical bedside', known as translational development. For example, in the past years we developed a gene therapy strategy for a rare genetic skin disease Netherton syndrome. We started with a series of studies including making vectors suitable for clinical use, correcting patients' skin cells, and then grew these gene-corrected cells in a 3D skin equivalent culture model to evaluate the efficiency of gene correction. Since the

results from these studies were encouraging, we further tested the durability of gene-corrected skin grafts and feasibility of the therapy strategy in the human:mouse skin graft model. The results confirmed that the skin grafts generated from gene-corrected cells could restore defective protein productions and reverse abnormal skin architecture to the normal. We presented these data to the Medicines and Healthcare Products Regulatory Agency (MHRA), an executive agency of the Department of Health and Social Care in the UK which is responsible for ensuring that medicines and medical devices work and are safe. This has directly led to initiation of translational developments and approvals for a first-in-man gene therapy clinical trial for Netherton syndrome.

We will launch new therapies for patients with these conditions using similar platform and pathway developed for Netherton syndrome in the project.

How will you look to maximise the outputs of this work?

The research outcomes will be disseminated via open-access publications in widely read, peer reviewed journals, oral and poster presentations at national and international meetings, and at public engagement events such as fundraising events. A close working relationship with our institute press office and the relevant patient support groups ensures that key messages from our work will be broadly disseminated.

Species and numbers of animals expected to be used

- Mice: 480

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use severe combined immunodeficiency (SCID) mice in our study. The reason for using immunodeficient mice is that being immuno-deficient, the mice do not reject human tissues. Since we will graft human skin sheet generated using human skin cells onto mice in the study to test therapeutic efficiency, the SCID mice will be the animal that meet the requirements.

We will use 2 to 3 months old mice in our studies, as they are adult mice and have large back (dorsum) surface for skin graft. In addition, if we graft human skin on 2 to 3 months old mice, allowing the graft growing for further 2 to 4 months, it means that mature adult mice (range in age from 3 - 6 months) will be used in the study. As mature adult mice are past the 'development' but not yet reach the 'aged' stage, their physical condition is stable with less incidence of age-related disease. All this will provide us a stable period with minimum unpredictable factors which may affect our study results.

Typically, what will be done to an animal used in your project?

Genetically altered mice known as SCID mice that can tolerate transplantation of human tissue will be used in our study. A piece of skin will be removed from the back of the mouse to make a wound and a human skin sheet generated from patients' or normal donor cells will be grafted onto the wound and secured with sutures. The typical surgery time will last 30 mins per mouse. The grafted skin sheet will grow on the mice for further 2 to 4 months, allowing them to blend with the surrounding mouse skin and form normal or disease-like human skin architecture. We will then test therapeutic strategies on the grafted mice by intravenous or intradermal injection or topical application of various drugs and therapeutic reagents. Following the treatment, the grafted human skin will be collected post-mortem and analysed to reveal whether the treatments are effective.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects include

i) The post-operation complications including dehydration, acute pain and infection. However, based on our previous studies, the symptoms of post-operation complications were short-lived (lasting less than 24 hours) and mild, and the incidence was less than 1%.

ii) Abnormal skin growth on grafted area. If grafts are generated using patients' cells with genetic conditions, abnormal skin growth is expected to be observed, such as mild blisters or exfoliated skin surface or thickening or scab skin. All this phenotype will last during the study. However, as the abnormal growth skin generated from patients' cells are relatively small (1-2 x 1-2 cm), localised and well blended with the surrounding mouse skin after wound healing, the abnormal engraftment does not affect normal behaviour of the animals. It has also been reported in a previous study that the engraftment generated from skin cells lacked nerve endings in the graft area for as long as 5 years post- grafting. Therefore, blisters or scab skin on the graft area would not cause pain and discomfort on grafted mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity for skin graft procedure is moderate and all mice subject to the procedure will have this severity. However, experimental mice will be anaesthetised. Prior to and post-operatively, analgesia will also be used to reduce the stress and pain caused by the surgery. According to our previous experimental record, 99% mice recovered (and have normal behaviours) 4-12 hours post-operatively.

Less than 1% mice had post-operative complications such as pain and delayed recovery. The majority reason for this was due to individual differences of mice.

The severity for injection is mild. This is because pain or suffering experienced by the animals following injection is only slight or transitory and minor. The animals following injection will return to a normal state, almost immediately. All mice subject to this procedure will have this severity

The severity for topical administration is mild as it is a non-invasive procedure. The topical drug will be made in non-irritant solution such as oil/cream or PBS/saline. The administration process may cause stress in the animals, but it is transitory and minor, and the treated animal can return to its normal state within a short time. All mice subject to this procedure will have this severity.

The severity of the project is therefore, graded as being of moderate (75%) and mild (25%).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To achieve a clinically-relevant method for skin disease therapy, it is essential to evaluate strategies used for these therapies in an appropriate preclinical model. There are in vitro 3D skin equivalent culture models which can be used to assess the efficacy of transgene expression and function.

However, this model cannot be used for observing the long-term effects of the transgene as cells can only survive for three to four weeks in the culture system. In addition, the in vitro system lacks a blood circulating system as seen in the human body and it cannot be used to assess trans-effect of transgenes.

Which non-animal alternatives did you consider for use in this project?

A skin equivalent 3D culture model, also known as organotypical culture, was considered for use in this project. In this model, human skin cells isolated from skin biopsies are firstly expanded and cultured as a skin sheet on a support matrix. After 2 to 3 days, the cell/matrix complex is lifted and further cultured at the air-liquid interface for 14 days to allow skin cells to grow and form a skin architecture in conditions that resemble the skin development in the human body.

Why were they not suitable?

The reason that the skin equivalent 3D culture model is not suitable for use as a pre-clinical study model is that i) it cannot be used for observing the long-term effects of the transgene as cells can only survive for 3 to 4 weeks in the culture system, and ii) it lacks a blood circulating system as seen in the human body, so it cannot be used to assess trans effect of transgenes.

Compared to the skin equivalent 3D culture model, the skin graft mouse model provides a unique opportunity to grow and keep cell survival for more than year. This long-term engraftment will allow us to analyse the durability of gene expression, the maintenance of skin barrier function and other safety issues including screening for transformational changes. This animal model also allows us to assess trans (generalised) effect of transgenes via blood circulating system and examine morphological changes on grafts. In addition, our previous studies have shown that the epidermal architecture of the grafts generated from normal or patients' keratinocytes and grown on this model resembled the original normal or patient's skin structure respectively.

We therefore chose the skin graft model mouse model for our study. It must be emphasised that before carrying out any study, we will test all materials and reagents in vitro first to confirm their efficacy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of mice to be used in the study has been estimated based on our previous studies. A typical experiment will be two groups, positive and negative group. Due to nearly 80% successful rate of skin graft from our previous studies, the study using protocol 1 needs 4 mice for each group, and the study using protocol 1+2 and 1+3 need 6 mice for each group respectively. In this way, we can ensure that we have enough successful grafted mice for performing further treatment and data analysis. In addition, the same experiments will be carried out at least two to three times with different patients' cells for evaluation of the efficacy of therapy.

Considering all these factors, the numbers of animals in five years have been estimated as following.

Protocol 1: 8 (mice) x 3 (times) x 5 (years) = 120 (mice)

Protocol 1+2: 12 (mice) x 3 (times) x 5 (years) = 180 (mice)

Protocol 1+3: 12 (mice) x 3 (times) x 5 (years) =180 (mice)

Total mice that will be used in the project in five-year time are 480.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will only carry out studies in live animals when the outcomes of in vitro or other laboratory bench- based studies are promising. In this way, we will make sure that each animal study is necessary. In addition, we will consider sharing controls between groups, between two repeat experiments. For topically-treated, an internal control will be used, i.e a skin biopsy will be taken from the mice before treatment. In this way, we will reduce the mice numbers used in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will reconfigure/modify and test vectors, small molecule drugs, siRNA, and other therapeutic reagents in the laboratory first, and will only test materials and reagents on the animals when we have promising outcomes from laboratory studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The human:mouse chimeric skin graft model will be chosen for our study. A piece of full skin with 30mm diameter is removed from the back of anaesthetised mice to make a graft bed. A bio-engineered skin prepared in advance in the laboratory is immediately grafted onto the wound bed to minimize further trauma. The graft area is then covered with denatured mouse skin and fixed with sutures to protect and hold the skin graft in place. Based on our previous experience, grafted mice recover and return to their normal status within 24 hours. Since this model does not involve massive invasive surgery and the suffering period in animals is transient and short, this animal model causes the least pain, suffering and distress on animals.

Why can’t you use animals that are less sentient?

Mice have been chosen as the experimental animal as they have a body environment with comparable complexity to the human. In addition, the use of gene therapy on mice is highly representative of the procedure in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The skin graft procedure (protocol 1) can cause moderate welfare costs to the animal, we will therefore, refine the following steps.

1. Before operation, analgesia will be used in mice to reduce the stress and pain caused by the surgery.

2. During operation, the graft bed will be covered with wet gauze between the interval of preparing graft bed and grafting to prevent dehydration. The grafting will be carried out immediately once the graft bed is ready to reduce the water loss.

3. After surgery, animals will be given post-operative analgesia to further reduce the stress and pain caused by the surgery.

4. The grafted mice will be closely monitored by the dedicated PIL every day in the first week post graft. Any abnormal finding will be recorded and discussed with NACWO/NVS and appropriate decisions will be made to reduce the suffering of the animals including humane killing by a Schedule 1 method.

5. Only PILs who have been trained and assessed as competent for operation techniques will be allowed to carry out the surgery

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The best practice guidance that we will follow including

i) Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 published by Home Office, UK.

ii) ARRIVE guidelines (2.0) published by NC3Rs.

iii) PREPARE: guidelines for planning animal research and testing published in the journal Lab Anim. 2018 Apr;52(2):135-141.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use the following ways to keep up to date with latest 3Rs.

1. Attending monthly Western Laboratory Advisory Group meeting to get latest news about 3Rs

2. Reading NC3Rs Newsletter about latest 3Rs news. This will be sent by NC3Rs Regional Programme officer and/or our university Biological Service Department

3. Regular discussions with the NACWO/NVS at our institution to review current approaches and whether there are any new 3Rs opportunities.

4. Attending events, symposiums and workshops hosted by NC3Rs/Society of Biology to keep abreast of 3Rs advances and approaches.

5. We will keep the contact information of our local NC3Rs Regional Programme Manager to having a direct route to get 3Rs advice, developments, and best practice.

37. The role of transposable elements in cichlid fish development and evolution

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Transposable elements, Gene silencing, Epigenetics, Adaptive Evolution, East African cichlids

Animal types	Life stages
Haplochromine cichlid fish of the East African Great Lakes.	adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the role of certain genes, termed transposable elements, in the evolution of East African cichlids.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to identify the genes underlying the evolution of animals. The knowledge of the genetic basis of evolution is relevant, for example, to predict how animals react and adapt to a fast changing environment. This understanding is most important nowadays given global climate change trends, which are increasingly having a deep negative effect

on biodiversity. This is particularly relevant for animals that live in a "closed" environment, from which they cannot migrate out of. Such is the case of fishes specialised to life in lakes.

Transposable elements are genes that exist in most animals. The special feature of these genes is that they can multiply and change their location within cells. As this change in location may damage other genes, yet another set of animal genes is dedicated to the control of the mobility of transposable elements. However, the mobility of transposable elements does not have only negative effects. In fact, some transposable elements may lose their ability to change location and become fixed in one location (termed "domesticated"), by evolving a useful function.

In the proposed research we will use a fascinating group of animal species, the cichlid fish of the East African Great Lakes, to investigate two aspects related to transposable elements:

1) the contribution of "domesticated" transposable elements to the evolution of these fishes; and

2) the genes that prevent the mobility of transposable elements.

What outputs do you think you will see at the end of this project?

Due to the presence of transposable elements in most animals and their ability to multiply, these genes are responsible for providing new genetic diversity that may, in some cases, be important for evolution. Genetic alterations of transposable elements and the genes that control them is required to understand what their function is in animals.

The results of the proposed work, using genetically altered animals, have the potential to result in several publications reviewed by other scientists. These publications will report findings related to the following main topics:

1) Identification of the genes controlling the mobility of transposable elements in cichlid fish, across several relevant parts of the body (including the reproductive organs and the brain), as well as in eggs and sperm.

2) Determination of whether the activity of transposable elements is affected in animals created by mating individuals of different species.

3) Identification and characterisation of "domesticated" transposable elements important for the evolution of these fishes.

Furthermore, it is likely that the data generated in our work will be useful to other researchers and lead to collaborative work. Importantly, our data will be deposited in publicly available, free repositories, so that other researchers can download it and use it to answer their own biological questions. This is also a reduction measure, as it should avoid repetition of the same work by other researchers.

These publications and collaborations are direct benefits resulting of this work, which will be likely produced within the timeframe of the license and shortly after. It is also likely that our publications will provide a substantial contribution on the medium-/long-term to biological research.

Who or what will benefit from these outputs, and how?

Our research is of a fundamental nature and will build important knowledge on the "domestication" of transposable elements and on the genes that control transposable elements. This will increase our understanding of nature and of the genes implicated in animal evolution. As transposable elements exist in most animals, our discoveries may be relevant to understand the evolution of other groups of animals, including mammals. As our project touches upon so many fundamental biological aspects, we expect to impact on several fields of the biological sciences, e.g. evolution, molecular biology, and genetics.

Given the fundamental nature of the proposed work, the research output is not likely to produce directly any product or method with commercial potential.

How will you look to maximise the outputs of this work?

I intend to present my results in international and local scientific conferences, or other scientific meetings. I will attend, at least, one major scientific conference per year on topics relevant to my proposed research. These conferences will provide opportunities to communicate my results broadly. Other persons involved in this work will also participate in dissemination efforts.

Our investigations and the data generated are expected to lead to new collaborations with other researchers. Our work will be published in free, public repositories online and in international scientific journals.

The genetically altered animals, or their eggs/sperm, will be openly available and shared with the scientific community, if requested and assuming all legal permissions are in place. Sharing animals worldwide will replace the creation of novel genetically altered animals, and reduce the total number of animals used in animal research.

Species and numbers of animals expected to be used

- Other fish:

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using East African cichlids of the African Great Lakes due to their unique and fascinating diversity of body sizes, shapes, diets, and behaviours. Although they vary in these ways, they do not vary much genetically, in fact being genetically very similar. The combination of these features make them, as a whole, a great system to pinpoint the genes underlying cichlid evolution.

It is beneficial for transposable elements to be active and multiply in the reproductive organs, eggs or sperm, or during early development, as this increases their chances of transmission to the next generation. Thus, the activity of transposable elements and of the genes that control them should be studied in the reproductive organs and during early development. In addition, we discovered genes that inhibit transposable element activity in the brain. This raises the interesting possibility that transposable elements are important for brain development and function. These exciting preliminary data justify the use of these parts of the body.

Typically, what will be done to an animal used in your project?

Creation of genetic alterations - fish eggs will be gently held and injected so as not to damage the egg. Immediately after, the eggs will be returned to aquaria. Injection of a batch of 25 eggs takes less than 20 minutes. Injections will be performed to create genetic alterations, meaning the addition or deletion of genetic material. Genetic alterations will be generated in transposable element genes, or genes involved in the control of transposable elements. These alterations will, in principle, not cause lethality.

Confirmation of the genetic alterations - After the creation of genetic alterations by injection of fish eggs, fish will be grown until they reach their juvenile stage. At this point, we will confirm the genetic alterations that we introduced. This will be done either by cutting a little portion of a fin, or by taking a swab from the fish. Swabs will be preferred to fin cutting, as recent data indicates that swabs provide the more refined method. Swabs gently collect skin cells and mucus, in a way that does not damage the scales and skin of the fish. When confirming genetic alterations using a portion of the fin, the amount of fin removed will not compromise swimming or any other aspects of normal fish physiology or behaviour, and taking the sample takes less than a minute. The cichlid fish we are using are large and robust.

Therefore, these procedures will be preferentially performed without anaesthesia, as this brings a higher risk of adverse effects.

Isolation of eggs and sperm - Adult fish will be anaesthetised and eggs and sperm will be obtained by applying gentle pressure on the sides of the fish.

What are the expected impacts and/or adverse effects for the animals during your project?

Our proposed procedures will impact the animals' health and welfare minimally and will not lead to long-lasting pain, suffering, or distress. The genetic alterations we propose to

introduce are highly unlikely to lead to any malformations or adverse effects. Some eggs may be damaged during the injection procedure, but these, whenever detected, will not be allowed to live past the stage where they are still very small and immature fish, before they start to feed independently. East African cichlids are large and will withstand well our proposed procedures. These procedures will be performed in the least amount of time possible and are not expected to lead to any long-term consequences or distress. After any procedure, animals will be monitored for an adequate amount of time to ensure recovery.

Animals are expected to remain healthy and normal and, should this not be the case, they will be humanely killed immediately to prevent any pain, suffering, or distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Fish: Mild severity: 100%.

What will happen to animals at the end of this project?

Killed
Used in other projects
Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Transposable elements are important genes for animal evolution. However, their roles in the evolution of East African cichlids remain unknown. We aim to understand if and how transposable elements influenced the evolution of diverse colours, shapes, behaviours, and dietary adaptations in these fishes. To conduct this investigation we need to generate cichlid fishes with genetic alterations.

Which non-animal alternatives did you consider for use in this project?

We have considered computer simulations and cultures of cells.

Why were they not suitable?

At the moment there are no alternative resources capable of recreating the natural development of fish. Likewise, eggs and sperm are created and maintained in a unique

and complex natural environment impossible to replicate in the laboratory. In order to fully understand animal evolution, we need to understand the role of transposable elements. To do so, we need to see how live animals with specific genetic alterations look and behave in a natural context. Other alternative systems, such as cell culture, or computer simulations, are not able to recreate the complex interactions that occur during development, between neighbouring cells and tissues. These interactions are most important for proper animal development. Thus, our aim cannot be accomplished without using animals.

We will use methods to collect mature sperm and eggs of cichlids, effectively bypassing the need to kill adult animals for the dissection of their reproductive organs. Eggs and sperm will be used as described, or frozen after their isolation. As these frozen eggs and sperm can be thawed and combined together in the laboratory to create new animals, their isolation will allow for the long-term conservation of our animals. Generation of animals in the laboratory will replace the continuous breeding of specific cichlids, when not in use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to produce animals with 3 distinct types of genetic alterations (maximum of 3000 animals):

1) We plan to produce up to 5 lines of animals with added genetic material. For their breeding, we expect to use a maximum of 1000 animals (5x200).

2) In addition, we will alter or delete genetic material of up to 5 genes required for preventing the activity of transposable elements. For their breeding we will need a maximum of 1000 animals (5x200).

3) We plan to alter or delete genetic material of up to 5 transposable elements in cichlids. We expect to need a maximum of 1000 animals (5x200) to breed these animals for the duration of the project.

Lastly, we aim to collect eggs and sperm from a maximum of 200 individuals (200 protocol 2).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Genetic alterations will be performed on a carefully chosen set of genes. At the beginning of this research programme, we used computational methods and available data to confirm that the candidate genes are active in the tissues of interest. In this programme of work, validation of our candidates will be performed using procedures not regulated by law. This careful consideration and preliminary analysis ensures our set of candidate genes are most relevant to the biological questions we aim to answer.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Throughout the duration of the project, we will use the minimum number of animals required to acquire satisfactory data. Experiments will be designed, conducted, and reported according to published guidelines (namely the PREPARE and ARRIVE guidelines).

Whenever an animal is culled, a variety of tissues will be extracted and frozen. As other colleagues may have an interest in tissues not necessarily relevant for the purpose of this project (e.g. scales, muscle, liver), tissue sharing will avoid culling of additional animals. Similarly, sharing data obtained from dissected tissues with colleagues will avoid culling of additional animals for repetition of these experiments.

Frozen eggs and sperm will ensure the long-term maintenance of fish lines. This practice will reduce the number of individuals bred, when they are not required, and will prevent duplication of experiments (i.e. re-generating animals with particular genetic alterations).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

I will use East African cichlid fish for the proposed work plan. As these fish species are large and robust, they can be effectively bred and grown in an aquaria environment. Moreover, these animals can tolerate the mild regulated procedures we propose to perform with the minimum amount of stress and pain.

The egg and sperm collection, the creation of genetic alterations and their confirmation will be conducted using current best practices and technology. These procedures are expected to cause little pain, suffering, and distress to the animals. Genetic alterations will

be created by injection of eggs, an immature life stage before the animals feed independently. None of our experiments will require animals to live in isolation.

Why can't you use animals that are less sentient?

The work proposed here takes advantage of the extreme diversity of shapes, sizes, colours, diets, and behaviours of East African cichlids, in spite of very close genetic similarity. The diversity of these cichlid fishes is unique amongst vertebrates and even amongst the entire animal kingdom. Therefore, other less sentient, or invertebrate, animals will not allow us to answer the same biological questions.

I will use whenever possible tissues obtained from immature life stages, i.e. from early development, before the animals reach the independent feeding stage. Tissue dissection and extraction, besides the methods to confirm the genetic alterations, will be exclusively performed in dead animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will incorporate new recommendations on animal welfare, as soon as possible after their publication.

Cichlid housing will be continuously improved to faithfully mimic the sandy areas rich in vegetation and hiding places characteristic of their natural environment. This will be achieved by introducing sand, artificial plants, clay pots, and plastic tunnels in the aquaria. Fish will be housed in groups, in order to decrease stress and aggression levels. Manifestations of aggression, including chasing, fighting, and injuries will be monitored daily by the scientific and animal care staff. These aggressive behaviours are normal to these species and not a product of captivity. If required, fish will be temporarily separated and groups redesigned to avoid further injury and aggression. Continuous monitoring of water parameters, food regimen, and breeding will ensure the wellbeing of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow the best practice guidelines available at www.nc3rs.org.uk. Experiments will be planned, documented, and reported according to published guidelines (namely the PREPARE and ARRIVE guidelines).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will follow the website of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, available at www.nc3rs.org.uk), and stay up-to-date with new information and new resources that become available. To achieve this, I have signed-up to the NC3Rs e-newsletter. In addition, named persons at my

establishment (i.e. the Named Animal Care and Welfare Officer, Named Veterinary Surgeon, and Named Information Officer) will inform us of relevant new information and resources, and will advise, together with the animal care staff, on the best ways of implementation.

38. Understanding the mechanisms and mediators involved in adverse pregnancy outcomes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

pregnancy morbidity, immune system, coagulation, placenta, imaging

Animal types	Life stages
Mice	adult, pregnant, embryo
Rats	adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to use a model of pregnancy to evaluate:

1. The role of inflammatory mediators and the clotting cascade in the development of pregnancy related disorders

2. Which cells are recruited and activated in pregnancy morbidity

3. The role of these different cell types (e.g. monocytes, macrophages, neutrophils, platelets, B cells, T cells, trophoblasts) in abnormal pregnancy outcomes

4. The use of non invasive imaging methods for early diagnosis and prevention of pregnancy complications

5. Novel cellular and molecular targets for therapy

6. A variety of agents and compounds as potential treatment for pregnancy complications

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

According to the WHO, 1500 women die from pregnancy or childbirth-related complications each day. In 12% of the cases, maternal death is caused by preeclampsia. In addition, among the 133 million babies who are born each year, almost 3 million die in the first week of life and a million in the following three weeks. Frequent pregnancy complications include unexplained recurrent early pregnancy loss, fetal death, or premature birth due to severe preeclampsia (high blood pressure), eclampsia (onset of seizures due to high blood pressure), intrauterine growth restriction (babies too small for their gestational age) or other consequences of placental insufficiency, a serious complication of pregnancy when the baby is unable to get nutrients and oxygen from the mother.

A developing baby goes through important growth throughout pregnancy— including in the final months and weeks. The brain, lungs, and liver need the final weeks of pregnancy to fully develop. Babies born too early (especially before 32 weeks) have higher rates of death and disability. Preterm birth is the leading cause of perinatal morbidity and mortality worldwide with figures from 2017 showing that preterm birth and low birth weight accounted for about 17% of infant deaths.Effective prevention or treatment of these pregnancy complications could significantly lower maternal and neonatal mortality and morbidity as well as health care costs. Unfortunately, there is no procedure or test that an obstetrician can employ to predict if the mother and the fetus are at risk for any of these pregnancy disorders. There is growing evidence of the association between coagulation, inflammation and activation of innate immunity with pregnancy complications. However, the mediators, receptors and cellular mechanisms responsible for adverse pregnancy outcomes remain unclear.

By using a model of pregnancy, we will be able to evaluate the role of inflammation, coagulation and other mediators in the development of pregnancy complications. In addition, we will be able to characterise effector cells and the potential mechanisms by which these cells influence the outcomes. We anticipate that our work will give important information on the mechanisms responsible for adverse pregnancy outcomes and will identify possible targets for therapy.

What outputs do you think you will see at the end of this project?

The project outlined will allow us, with the help of this model, to develop knowledge on the pathogenesis of pregnancy-related disorders in particular the role of mediators, receptors and effector cells. Novel imaging techniques may help us to better recognise mechanisms, diagnose and quantify the risk of pregnancy complications and to improve current interventions. Data from this project may allow us to more efficiently diagnose and treat patients at higher risk. We anticipate that our work will give important information on the mechanisms responsible for adverse pregnancy outcomes and will help us identify possible targets for therapy. This knowledge will help us develop the appropriate treatment to patients based on more quantitative and objective measures.

Who or what will benefit from these outputs, and how?

The animal models will allow us the identification of the mediators responsible for adverse pregnancy outcomes and targets for prevention and therapy. The expected immediate benefits of this research are the ability to make an informed decision whether to test the new agent in humans, or to abandon the agent, or return to the in vitro or chemical laboratory for further modification. If any of the targets identified in the different mouse models of pregnancy complications are present in humans, they could be effectively used to detect pregnancy complications before they manifest and develop new therapies to improve the health of mother and unborn child.

How will you look to maximise the outputs of this work?

We will closely collaborate with industrial and university partners. We will disseminate the results of this work at conferences and workshops and publish in peer reviewed national and internal scientific journals. In addition, we will organise workshops at KCL where we will share our results to KCL and external academics interested in this work.

Tissues will be harvested for use in histology. These tissues will be kept for re-probing or further quantification. Imaging data will be compiled in an imaging library and shared with other investigators.

Species and numbers of animals expected to be used

- Mice: 1000
- Rats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Considerable number of features of pregnancy are similar in humans and mice, therefore, mice constitute a good animal model. Mice are quick to reach sexual maturity (7-8 weeks) and they have a short reproductive recovery (less than a week) with a short intergenerational interval. Mice have short gestational period (≈ 20 days) and the reproductive tract is relatively small, thus it is possible to analyze the course of pregnancy in exquisite detail with minimum or no harm.

Typically, what will be done to an animal used in your project?

The typical experience for an animal includes:

1) Animals will be mated by conventional breeding methods to induce pregnancy

2) An agent which is likely to perturb pregnancy (i.e. antibody) will be administered

3) Blood collection (optional)

4) Administration of a drug or agent to potentially revert pregnancy morbidity (optional)

5) Imaging in vivo (by MRI, PET, SPECT, etc) under anaesthesia with or without administration of contrast agents (optional)

6) Food withdrawal for up to 18h (optional)

7) Schedule 1 culling

What are the expected impacts and/or adverse effects for the animals during your project?

In the mouse model of pregnancy morbidity, around 40% of the embryos die in utero. The dead embryos quickly undergo resorption and the remaining 60% make it to term. No signs of maternal health compromise are observed in this model.

All agents will be administered at doses known to be non-toxic, based on experience and dosages reported in the literature, and at volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

Besides the momentarily discomfort of the injections no distress or harm is inflicted by the procedures.

Moderate severity may be reached by 20% of animals to which a microosmotic pump is inserted. This procedure carries the risk of wound breakdown and infection.

Regular monitoring of the wound and the animal behavior will be performed.

Deaths resulting from anaesthesia or surgical complications are uncommon (<1%) and will be minimised by correct dosing of anaesthetics, by accurate weighing and by maintenance of body temperature during and post procedure e.g. use of heat pads.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 80% mild severity

20% moderate severity

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To date, there are no alternate systems such as computer models, cell culture or in vitro assays to adequately replace animal models for pregnancy.

We require the use of animals because:

1. Our project needs mammals of the order eutherians (placental mammals like humans) to allow us a correlation of our results to humans. The study of the physiological dynamics between mother and offspring requires an intact animal and are essential to the understanding of pregnancy complications

2. Data generated from this body of work may be used to inform whether to go forward to human clinical applications. Regulatory agencies require animal data to demonstrate safety and efficacy before therapeutics (that can be validated through imaging) or molecular imaging agents can enter human trials.

3. To validate the mode of action new/improved agents or molecular imaging compounds, experiments are required that cannot be conducted in humans for ethical and scientific reasons (e.g. contrast agent distribution requires intact physiological barriers and excretion mechanisms).

4. Bio-distribution in whole organisms (i.e. tracking the injected agents route/ accumulation and excretion through the body), with intact biological barriers and excretion mechanisms, is key to clinical use.

Which non-animal alternatives did you consider for use in this project?

Pregnancy is a phenomenon that only occurs in animals. Our project needs mammals of the order eutherians (placental mammals like humans) to allow us a correlation of our results to humans.

The study of the physiological dynamics between mother and offspring requires an intact animal and are essential to the understanding of pregnancy complications and to date, there are no alternate systems such as computer models, cell culture or in vitro assays to adequately replace animal models for pregnancy complications.

Whenever, and if, possible (i.e. toxicity, binding or stability studies) in vitro experiments will be performed using cells and ex vivo tissues to minimise unnecessary harms.

Why were they not suitable?

If we are to further investigate the causes of pregnancy complications, the study of the physiological dynamics between dam and offspring requires an intact animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals required based on published information on the proposed model. Whenever possible, we have looked at our previous data for reproducibility and compared to what is available in the literature.

A typical study might be planned as follows:

- For testing a new agent in mice, we estimate animal numbers using a power calculation (two-tailed t- test, α=0.05, power=0.9), yielding N=6 animals/group. To account for complications with anaesthesia, interventions or treatments, we would plan to investigate 10 animals/group. If difference between experimental and control groups, is expected to be large (>1 order of magnitude change, e.g.in the case of using effective blocking substances/treatment), or found to be so by pilot experiments, statistical significance may be achieved with fewer animals/group. However, we will not use less than 3 animals per group to comply with generally accepted scientific reproducibility criteria in the field.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1. We will limit the group size to the minimum needed to obtain statistically significant data. Sample sizes will be set using power analysis. For the majority of the experiments we will aim to have a significance of 5% and a power of 90%

2. Wherever possible, we will perform multiple experiments simultaneously so that the same control group can be used for all experiments

3. Wherever possible, we will try to collect as many different tissues from each animal so that additional animals are not needed

4. Wherever possible, we will try to use new instrumentation/methodology that improves precision and reduces the number of animals. For example, following the observations of Seaborn et al. (Promoting the "3Rs" Principle in Developmental Biology with Early and Convenient Diagnosis of Pregnancy in Mice J Reprod Dev 2011), we will address Reduction and Refinement in the use of mice by using maternal percentage of weight increase at gestational day 8 to diagnose pregnancy in mice. Body weight increase is a better indicator than vaginal plug, which is associated with false negative and positive rates. Ultrasonography may be accurately used as an alternative non-invasive technique for pregnancy diagnosis in the mouse from very early stages of gestation (day 5.5). These methods allow pregnancy detection before midgestation and propose an ethically sound approach to experimental animal use by optimizing the number of mice used and refining animal manipulation.

5. Non invasive sequential imaging of individual mice at differing time points (longitudinal study) will enable each animal to act as its own control. Previously animals were sequentially sacrificed at pre- determined time points to enable sequential data to be collected. In vivo imaging will diminish the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible, we will perform multiple experiments simultaneously so that the same control group can be used for all experiments and will try to collect as many different tissues from each animal so that additional animals are not needed.

When a new agent is used for the first time in mice the concentration of the agent in the blood will be used an indicator to calibrate the dosage. The minimum number of mice will be used in a pilot study (n=6). Animals will be treated with the new compound and small amounts of blood will be drawn from a superficial vein, with or without local anaesthesia, to measure the concentration of the agent in the plasma.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In this project, we will use a model of normal pregnancy obtained by conventional breeding methods.

Species: Mice and rats are the species of least neurophysiological sensitivity that provide the capability to support the development of a normal pregnancy and the minimum size compatible with the scale of resolution/movement associated with the imaging technique. Resolution of whole-body imaging techniques is of the order of 0.1-1mm. The considerable larger size of rats compared to mice make them more suitable for diagnostic imaging.

Only moderate severity may be reached by those animals to which a micro-osmotic pump is inserted. In these cases, close monitoring, anesthesia and pain relief will be provided.

Why can’t you use animals that are less sentient?

A considerable number of features of pregnancy are similar in humans and rodents and, therefore, mice for example constitute a good animal model. Mice are quick to reach sexual maturity (7- 8 weeks) and they have a short reproductive recovery (less than a week) with a short intergenerational interval. Mice have short gestational period (≈ 20 days) and the reproductive tract is relatively small, thus it is possible to analyze the course of pregnancy in exquisite detail. There is not a less sentient animal that can be used to replicate elements of human pregnancy.

The considerable larger size of rats compared to mice make them more suitable for diagnostic imaging and therefore, rats may be used for some of the experiments whenever necessary.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We do not anticipate animal distress or suffering as the protocols we will use are mild. Animals will be monitored daily at early stages and at least three times weekly once early clinical signs have developed.

Only moderate severity may be reached by those animals to which a microosmotic pump is inserted. In these cases, pain management will be administered under the close consultation with the NVS.

All agents will be administered at doses known to be non-toxic, based on experience and dosages reported in the literature, and at volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

Inhalation anaesthesia will be used wherever possible to minimise transient pain and distress, e.g. mini-pump insertion, during imaging. In addition, full recovery between periods of anaesthesia, rehydration during long imaging sessions, respiration/cardiac function monitoring, body temperature monitoring/maintenance will be conducive to animal wellbeing.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These includes:

1) The Responsibility in the use of animals in bioscience research produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

2) The Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

3) Accepted limits of volumes and frequencies when administrating compounds and anaesthesia (Appendix 1a in Action Plan section).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

Additionally, we have direct support and contact with an NC3R's regional Programme Manager who supports the application of the 3Rs at King's College London and the Francis Crick Institute and is a member of the Policy and Outreach Group. This includes providing expert advice and coordinating the sharing of best practice.

39. Understanding trait variation in migratory birds

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Animal Migration, Ecology, Breeding biology, Ornithology, Stable isotopes

Animal types	Life stages
Brent goose Branta bernicla	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Studied for 1000s of years, migration remains one of nature's most puzzling phenomena. This project seeks to understand the causes and consequences of variation in foraging and movement behaviours in migratory birds and how these in turn link to fitness and drive changes in demography.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Migrants are amongst the most threatened of all bird groups, climate change, habitat change and increasing urbanisation are all thought to play a role but our understanding of

their importance and how they might interact is limited. This is in part because, it is exceptionally difficult to identify where and when in the annual cycle different migratory populations are regulated. To progress we need to be able to map individual responses to changing environments onto population processes and to do this we need to be able to track individuals throughout their migratory journeys. My group are working on a series of study systems that are allowing us to start to piece together the links between the drivers of change in migratory species and this licence will enable us to use forensic approaches to infer habitat choices and migratory routes.

What outputs do you think you will see at the end of this project?

In general terms, the tracking and stable isotope data will contribute to the basic research on obligate and partial migration route of multiple species, many of which have long remained unclear. More specifically, the recent technological advancements in the application of stable isotope techniques this project will provide empirical migration data to the ornithological and scientific community. By integrating stable isotope data, environmental data, behavioural data and morphology, this project will enhance our understanding of partial migration and the processes that drive it. The novel insights the project is likely to generate will be of interest to a range of scientists working on migration, ornithologists (both academics and enthusiasts) and conservationists.

In short term, the major outputs of this project will be the production of a PhD thesis and peer-reviewed scientific papers. The former will be fundamental to the career development of young scientists and both will enhance our knowledge of the phenomenon. These outputs will be largely targeted at the academic community, but also hobby ornithologists, bird ringers and managers (particularly if we can develop morphometric assays for identifying the migratory and non-migratory components of populations).

In medium and long term, output from this project is expected in influence and guide further research in the largely under-studied field of (1) avian partial migration and (2) ecological conditions that may shape migration strategies. Moreover, understanding the links between migratory behaviour, body size measurements and other individuals of the same species has the potential to inform conservation strategy for threatened migratory species (e.g. which components of the population are at most risk and where in the annual cycle this risk is greatest).

Who or what will benefit from these outputs, and how?

The major beneficiaries are likely to be other scientists with an interest in intra-population variation, the ecological niche and migration. However as outlined above, information on habitat use of geese and shorebirds may help conservation managers in the areas where we catch them. Elements of basic research on migratory origins and distinguishing resident/migrating individuals is also expected to contribute to future studies into the origins of migratory species, population structure within migratory species and in turn conservation planning. Tracking the migration origin/destinations of individuals will enable

much more precise and targeted conservation plans that can cover protection/management of the habitats required by both the migratory and resident subsets of the population. This will be particularly important for shorebirds in Northern Ireland that are currently undergoing population decline such as the Oystercatcher which is listed as Near Threatened on the IUCN Red List.

How will you look to maximise the outputs of this work?

My research team have an excellent record of data analysis and publication. As such I see this as the primary route for dissemination. Work will be presented at conferences and workshops (including unsuccessful investigations) and publications will be shared in accessible form (e.g. tweetable abstracts) on multiple social media channels (Twitter, Instagram, Facebook etc). The university also has an excellent media team for reaching the more conventional media outlets and some of our proposed work is likely to be of interest here.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The research will investigate what drives variation in migration behaviour and its consequences. We have chosen 9 different species of wild bird. The species have been chosen according to their migratory behaviour in order to allow us to test the questions of interest. As such, we have long distance migrants, short distance migrants, species where migration distance varies among individuals and species where there are both resident and migrant individuals. We use adult individuals as they are the ones that make the decisions of when and how to migrate, as breeders is it also possible (in some instances) to measure the reproductive benefits or costs associated with different behaviours.

Typically, what will be done to an animal used in your project?

All birds will have feathers and a small amount of blood collected, and some will have a small piece of toenail clipped. We will measure chemical fingerprints in these samples in order to infer the diets and migratory origins of individuals. The entire procedure will typically take less than 5 minutes and birds will be released back into the wild after it is complete. We would plan to collect these materials from around 1600 individuals across all species over the course of the project (species breakdowns detailed below). Any individuals retrapped during the course of the project will not be resampled. These animals will be identified via individual rings fitted at the time of original capture.

What are the expected impacts and/or adverse effects for the animals during your project?

There may be a small amount of pain during blood collection or the collection of feathers, but these are likely to be transient. Birds may show panting, gaping, closing eyes, fluffing up feathers or immobility butt usually return to normal behaviour within a few minutes of release.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are very mild for all animals.

What will happen to animals at the end of this project?

- Set free
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This is a study of a wild behaviour, there are no appropriate models available

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives to consider that can address our questions.

Why were they not suitable?

NA

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For all species, sample sizes in excess of 100 individuals are needed to account for variation among life histories (e.g. sex and age class) and migration behaviour, but vary as such life history variables are not consistent across species.

For Brent geese the number is higher than other species because we need to have data from sufficient individuals returning with offspring. Breeding is highly variable on an annual basis (in some years less than 1% of individuals breed successfully). In order to account for the exceptionally high yearly variation, larger sample sizes across five years is required to answer the research questions detailed below in later parts of the report.

For oystercatchers and redshanks, numbers are based on the frequency of alternate migration strategies and size of flocks in catchable areas.

For the other species (Blackcap, Robin, Blackbird, Chiffchaff, Goldfinch and Greenfinch) the numbers are estimated numbers to secure sufficient sample sizes of both resident and migratory individuals (while accounting for additional variables such as sex and age) based on their proportions within the population in the local area of capture.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers of individuals are estimated based on the uncertainties outlined above, along with the necessary sample sizes needed to provide enough power to handle multiple explanatory variables (e.g. sex, body size, habitat, body condition, date etc) in addition to those of interest (diet and migratory behaviour).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Sample sizes will be large in the early stages of the project. However, once the first data sets start to come online, it will be possible to run pilot analyses in order to estimate whether subsequent sample sizes can be reduced.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Each species represents a route to testing a different aspect of the hypothesis. There are no model alternatives and the sample collection (blood, feathers and claws) is the least invasive way of accessing the forensic information on diet and migratory origins. Alternative methods (such as the collection of muscle tissue) would be more painful, stressful and more likely now to cause lasting harm to the animals.

Why can't you use animals that are less sentient?

The questions are focussed on the behaviour of adult animals in the wild. Thus, immature life stages would be inappropriate. Likewise less sentient models or terminally anaesthetised animals would not enable us to address the questions of interest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The effect of the procedure is likely to be transient and the approaches are well-established. But all animals will be marked offering the opportunity to assess the behaviour of a subset of individuals post release.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to consult the species/taxon focussed literature and the relevant Home Office wildlife research pages for new approaches and guidance. Likewise there will be ongoing discussions about approaches with colleagues, veterinary teams, technicians and the HO inspector for ongoing advice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep abreast of advances via reading the recent scientific literature, discussions with colleagues and discussions with the HO inspector and university support teams. Any new guidance that emerges will be incorporated into the procedures and the licence will be amended accordingly.

40. Developing flare-responsive intra-articular steroid injection

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Glucocorticoid, hydrogel, rheumatoid arthritis, inflammation, metabolism

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Intra-articular injections of therapeutic glucocorticoids (GCs) are widely used in the treatment of chronic inflammatory arthritis to suppress inflammation and joint destruction.

This proposal has two core aims. The first is to test whether specially designed gels known as 'hydrogels' are able to extend the release and therapeutic duration of intra-articular GC injections relative to standard formulations used for this purpose.

The second is to test whether we can utilise the natural 'metabolism' and inflammatory targeted activation of inactive GCs within the joint to reduce their off target side effects, to prevent local bone loss and cartilage damage.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Intra-articular glucocorticoids (GCs) injections are routinely administered to patients with joint pain and inflammation, including rheumatoid arthritis, with a predicted global market value of 5.7 billion by 2026. However, the intra-articular delivery of therapeutic GCs such as methylprednisolone suffers from limitations that restrict efficacy. These include short therapeutic duration (up to 4 weeks) and recent high profile reports of localised bone loss and cartilage damage that exacerbate joint destruction. Therefore, there is an unmet need to develop better tolerated, longer lasting injectable GCs. This project aims to address this problem using novel GC loaded hydrogels

What outputs do you think you will see at the end of this project?

This project will provide new knowledge on the delivery of therapeutic glucocorticoids within novel gel formulations that we predict will extend their duration of action, therapeutic efficacy and safety profile in rheumatoid arthritis. This knowledge will be shared with the wider research community in the form of publications in scientific journals and presentations at conferences. Furthermore, the project will inform further funding applications to support and inform the development of new drugs and clinical trials with the goal to help patients with rheumatoid arthritis.

Who or what will benefit from these outputs, and how?

In the short-term, the improved understanding of whether slow release metabolism activated steroids are effective in the treatment of rheumatoid arthritis will benefit many research groups exploring steroid application in human inflammatory disease. This will include fundamental insights about the importance of steroid metabolism on the actions of therapeutic glucocorticoids and the efficacy of gel delivery to extend their therapeutic application and safety. These findings will be shared with the research community over the next 1-3 years of the project.

In the medium-term, these data will guide the design of future clinical trials examining glucocorticoid delivery in these gel formulations. These benefits of our research are expected to manifest in 5-8 years.

In the long-term, the development of novel treatment approaches to safely prevent side effects, and increase the anti-inflammatory efficacy of therapeutic intra-articular glucocorticoid injections would mean that patients with rheumatoid arthritis are the major future beneficiary of this research. The clinical benefits may be transferrable to other patient groups with inflammatory arthritis where there is common underlying pathophysiology and are expected to be realised over 8-10 years.

How will you look to maximise the outputs of this work?

This research fits within the interest of major research centres of excellence studying inflammation and aging and rheumatoid arthritis treatment and pathophysiology. Through our current links and involvement with these centres, we would ensure wider dissemination of findings to research organisations working in this field, as well as interested patient engagement groups. All findings will be routinely published to inform methodological approaches in appropriate journals and academic conferences regardless of experimental outcome.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Murine models of polyarthritis arthritis have been extensively studied and validated to model human disease where they share relevant immune mechanisms of human disease. Crucially we have selected models that present with the articular and extra-articular manifestations of inflammatory joint disease that we aim to prevent. Whilst alternative models of monoarthritis were considered in this setting, their application is not suitable in this instance based on the requirement for additional intra-articular injection to induce disease activity that would complicate interpretation of intra-articular therapeutics interventions, and increase experimental variability between animals and require significantly greater experimental animal numbers to detect therapeutic efficacy. This is coupled with a limited understanding of how intra-articulate glucocorticoids mediate their anti-inflammatory effects in this model, requiring additional validation and supporting studies. In contrast, the proposed models of polyarthritis more accurately model the rheumatoid arthritis disease setting where the therapeutic steroids proposed in this study are aiming to be employed. Further key points that make the proposed murine models of inflammatory arthritis the ideal in vivo organism to examine this include that the methods proposed have been validated and optimised by my research team. Here, murine models are recognised as showing the least degree of neurophysiological sensitivity, pain, suffering, distress and lasting harm for this nature of research. Consequently, exploring novel treatments in these well- established murine models before humans reduces risks of harm to humans.

The study will utilise the TNF-transgenic murine models of polyarthritis, which develops spontaneous and reproducible disease. Producing and maintaining these animals will employ breeding techniques that involve all stages of the life cycle. Research on the therapeutic actions of GCs administered by intra-articular injection and their role in

suppressing disease activity and preventing joint destruction requires animals that have completed development and we will therefore use adult mice only.

Typically, what will be done to an animal used in your project?

Breeding genetically altered animals involves mating and reproduction of mice by natural means. Small tissue samples may be taken infrequently to track the genetic alterations present in each animal.

TNF-tg mice will receive up to two intra-articular injections (given at least 10 days apart) of either saline or injectable therapeutic glucocorticoids that aim to suppress inflammation and prevent localised joint damage. Animals will be monitored for joint inflammation at regular intervals three times a week using score sheets to measure number of joints affects and severity of inflammation. Blood sampling may be taken on no more than three occasions to assess systemic exposure to GCs. Further samples and data will be collected during non-recovery general anaesthesia or after death.

What are the expected impacts and/or adverse effects for the animals during your project?

The TNF-transgenic model of poly arthritis develops progressive joint inflammation. From 8 and 12 weeks of age TNF-transgenic animals show reduced weight gain, reduced mobility and evidence of pain behaviour (including reduced weight bearing). These are monitored and scored three time weekly and where evident are indications to initiate pain relief. For the TNF-tg model of inflammation, arthritis will be maintained for no longer than 28 days to assess anti-inflammatory properties and steroid release over this duration. Any animals developing persistent discomfort (despite pain relief) beyond moderate changes, which exceed predetermined scoring criteria, will be humanely killed. If an animal loses weight rapidly, stops eating or displays persistent abnormal behaviour they will be humanely killed.

Intra-articular injections of steroids under anesthesia are predicted to cause mild discomfort and pain for up to 72 hours post injection. Evidence of pain and reduced weight bearing will be managed with daily buprenorphine pain relief. Evidence of persistent pain and loss of weight bearing beyond 72 hours will be humanely killed.

Injections, and blood sampling, may cause mild discomfort that is short-lasting. In exceptional circumstances when more significant discomfort is observed in an animal with these procedures, it will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 95% of animals will develop arthritis and so are expected to be of a moderate severity, with the remainder of animals under this PPL being of a mild severity. All experimental animals developing polyarthritis are expected to experience discomfort that will not exceed moderate severity. The majority of animals undergoing breeding procedures, injections and blood sampling may experience short-lasting and mild discomfort.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

My research group routinely use human ex vivo tissue biopsies and primary cell cultures to model the rheumatoid arthritis joint environment and examine and test our research questions. Using these resources we have performed the initial testing and validation of the novel intra-articular gel formulations being examined in this proposal. These methods have been instrumental in demonstrating the anti-inflammatory efficacy and bio-compatibility of our intra-articular glucocorticoid formulations for use in diseases such as rheumatoid arthritis. However, based on these findings there is now a strong impetus to ascertain whether these glucocorticoid interventions would represent an effective way of to protect suppress inflammation and joint destruction in a human disease setting. Unfortunately, we cannot proceed down this pathway until these concepts have been validated in an in vivo model prior to entering clinical trials and being tested in patient cohorts.

Which non-animal alternatives did you consider for use in this project?

We have explored conducting studies in human ex vivo biopsies of rheumatoid synovium and using cell culture models from key target immune and stromal cell populations.

Why were they not suitable?

Human cell culture models of the key cell populations found within the joint and ex vivo tissue models will be utilised within this project where possible to study cellular and molecular processes and reduce the number of animals required for research. However, these in vitro and ex vivo models cannot adequately replicate the complex interactions between multiple organ systems that occurs in the rheumatoid joint in a living organism. Moreover, tissue culture models cannot adequately replicate the specific environment that act on these cell populations during movement. Animal models are therefore needed to replicate human disease faithfully and address our research question with relevance to

clinical medicine. They are also an essential component required for preclinical testing of therapeutics designed to treat rheumatoid arthritis and are necessary for examining the interventions outlined within this proposal in future clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Experimental group sizes have been calculated using existing in house preliminary data in TNF-Tg mice receiving traditional therapeutic glucocorticoids to inform statistical calculations on the lowest required number of animals to detect meaningful scientific results. During these projects we developed first-hand experience in delivery and dosing of therapeutic glucocorticoids required to suppress disease activity and joint destruction in these models. The planned pilot study adheres to a design that is embedded in the experimental protocol. This means that data from animals used in the pilot study can contribute to final result analysis, rather than duplicating use of animals. Colony sizes for breeding animals have been determined based on existing breeding protocols for the TNF-transgenic mouse model that are ongoing within our institution and informed by published research reporting welfare guidance on their maintenance. These are under continual review with expert advice from both the NACWO and NVS to ensure that numbers required for maintenance and breeding new transgenic lines are kept to a minimum.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experiment is devised in a 2x3 factorial design, allowing efficient use of data generated from control groups and thereby minimising the number of animals required. Care has been taken throughout the experimental design and with selection of outcome measures to reduce unwarranted variability as much as possible. Steps to this effect include randomisation, blinding, or selecting muscle mass as a highly sensitive outcome measure. This will reduce the number of animals required to identify meaningful results. In conjunction with pilot studies, we have optimised methods of tissue and data collection from individual animals ensuring that these numbers are sufficient to address all of our research goals whilst providing biologically significant results that would not require further experimental repeats. Throughout these protocols full factorial designs will be used where applicable, with routine use of blocking where appropriate.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In house resources and expertise will reduce the number of animals needed to deliver this project. C57Bl6 mice with appropriate genetic alterations for this protocol are already available in house, reducing the need to establish new genetic lines or backcrossing animals. Breeding practices will adhere to local standard operating procedures and national NC3Rs guidance to maximise efficiency.Our research group has previously optimised the data and tissue collection methods required to deliver this project. This avoids need for further optimisation, reduces variability and ensures that high quality data is obtained from every animal. Where operator-dependent measurement variation is possible, we will take precautions to perform measurements according to standard protocols and by the same operator wherever feasible. The application of the anti-TNF biological therapy 'infliximab' has been utilised for all TNF-transgenic breeding animals to fully suppress disease activity, increase welfare, breeding lifespan and litter sizes. These allow smaller breeding colony sizes for these TNF-tg animals. Finally, we will conduct a pilot study to confirm that our estimates for the minimum required animal number is indeed correct, prior to embarking on the full-scale project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The TNF-tg murine model of polyarthritis will be examined in this project. Importantly, this model displays the articular and extra-articular manifestations such as bone damage, cartilage erosion and joint inflammation that our interventions are aiming to prevent. A model of polyarthritis rather than a model of monoarthritis has been chosen for this study for several important reasons. Firstly, the intra- articular injections required to initiate monoarthritis are themselves a potential cause of local joint damage at the injection site that would act as an additional confounder in delineating the impact of subsequent therapeutic glucocorticoid intra-articular injection. Secondly, the therapeutic efficacy and off target effects of glucocorticoids in bone and cartilage that we are investigating in this study have yet to be fully characterised in models of monoarthritis. Consequently, additional experimental animals would be required to establish effective therapeutic dosing ranges and characterise effects on bone and cartilage relative to the polyarthritis models where these experiments have already been performed.

Thirdly, this study aims to model and examine the efficacy of intra-articular glucocorticoids in human rheumatoid arthritis where features of disease and their response to glucocorticoids differ from mono- arthritic diseases such as osteoarthritis. In particular, leukocyte recruitment is known to differ, and therefore their response to metabolism activated steroids remains an unknown, meaning further validation and optimisation would be required for these models. Finally, whilst experimental model choice is limited due to scientific requirements, the polyarthritis models have been refined to ensure they cause the minimum possible pain and distress.

This species and procedures have been chosen as they represent the lowest animal models with a musculoskeletal system in which it is possible to study the complex arthritic joint and interventions designed to prevent joint destruction that reflect human disease. All methods and protocols applied within this proposal have undergone rigorous prior optimisation, ensuring we are able to use pre- optimised doses of glucocorticoids within the lowest acceptable therapeutic ranges that are well tolerated, and mean that all inflammatory models will run for the shortest possible duration to minimise suffering. Throughout, behavioural nociceptive (pain) testing in conscious unrestrained animals

provides an important and strong indicator of changes in pain thresholds and provide clear indications for the application of pain relief that minimise suffering in experimental animals.

Why can’t you use animals that are less sentient?

Less sentient animals and embryos pre E14.5 do not possess the same sort of skeletal structure that composes the joints, and often their vascular tree and immune system do not fully represent that of humans. Small rodents are the lowest mammals that can be used to recapitulate the human immune systems response to joint inflammation. The development of polyarthritis, modelling human disease, is a gradual process occurring over several weeks. As such these models are not suited to terminally anaesthetised animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The application of the anti-TNF biological therapy 'infliximab' has been utilised for all TNF-transgenic breeding animals to fully suppress disease activity, increase welfare, breeding lifespan and litter sizes. These allow smaller breeding colony sizes and improved animal welfare. For the breeding of TNF-tg animals, we use heterozygous TNF-tg males paired with wild type females. Consequently, pregnant females do not have the burden of pregnancy plus arthritis. Each experimental model will be monitored daily following intervention and mice will be assessed for any signs of distress such as pain and inability to feed. Therapeutic substances identified within this proposal will be tested in a small pilot study and the mice monitored daily for signs of distress. Humane endpoints will be strictly adhered to at all times. To support this we have extensively refined the scoring system for each individual polyarthritis model to capture the specific aspects of each arthritis

phenotype, ensuring clear and consistent analgesia and humane endpoints. In particular, we have shown that opiate pain relief using buprenorphine does not interfere with disease progression and therapeutic glucocorticoid responses in this model.

We have also made refinements to the housing of the animals to cater for any disability arising from arthritis - including soft flooring, non-tangling nesting material, long spouts on water bottles, food on the cage floor.

These aspects will be systemically reviewed following each experiment to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the subsequent experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All experiments will abide by local best practice procedures for breeding, maintenance and surveillance of animals. National guidance by the Laboratory Animal Science Association, National Centre for the Replacement, Reduction and Refinement of Animal Research, and the Royal Society for the Prevention of Cruelty to Animals was used in the development of this protocol, including:

Avoiding mortality in animal research and testing (https://view.pagetiger.com/RSPCAAvoidingMortalityResearchReport/RSPCA)

Blood sampling techniques, frequencies and volumes (https://nc3rs.org.uk/3rs-resources/blood- sampling, https://www.nc3rs.org.uk/blood-sample-volumes)

Genetically altered mice (https://nc3rs.org.uk/gamice)

Animal handling and husbandry (https://nc3rs.org.uk/3rs-resources)

The PREPARE guidelines (https://norecopa.no/prepare) informed the project planning phase, including engagement of relevant stakeholders and implementation of quality controls. Furthermore, the ARRIVE guidelines have been considered to ensure that results can be communicated with the highest scientific rigour. Specific guidelines in regards to the welfare and maintenance of rodent models of polyarthritis by Hawkins et al, have been utilised in the design and implementation of this proposal:

Hawkins, P., Armstrong, R., Boden, T. et al. Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacol 23, 131–150 (2015). https://doi.org/10.1007/s10787-

015-0241-4

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to engage with Institutional efforts to promote the 3Rs and workshops; and receive the NC3Rs newsletter.

41. Investigating mechanisms and novel therapeutics for critical limb ischaemia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Blood, Vessels, Development, Mechanisms, Therapy

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

1.Investigate how blood vessel develop following a blockage in arteries supplying blood to the legs leading to a poor blood supply (limb ischaemia) and low levels of oxygen reaching the tissues in the legs.

2.Investigate treatment strategies, using cells and molecules, to promote the development of new blood vessels or help to enlarge existing blood vessels in order to overcome the lack of blood flow in an ischaemic limb

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fatty deposits (atherosclerosis) in the arteries of the legs (peripheral arterial disease) affects 20% of individuals over 75 and can cause narrowing of the blood vessel, creating a poor blood supply to the legs which reduces the supply of oxygen to tissues in the affected leg. When severe enough, this can result in a condition called critical limb ischaemia (CLI) that manifests in pain, ulceration and gangrene. The quality of life in patients with CLI is similar to those with terminal cancer, with ~33% of patients with CLI that cannot be helped by conventional treatments requiring amputation of the affected limb. A limb is amputated every 2hrs in the UK as a result of CLI.

What outputs do you think you will see at the end of this project?

1. A better understand of the mechanisms that regulate blood vessel development in legs with poor blood supply (ischaemia) and in particular the relationship between tissue resident and circulating cells in promoting blood vessel development in the ischaemic limb.

2. Novel therapeutic targets will come from biochemical and cell analysis, with comparisons of blood and tissues obtained from patients with a critically ischaemic limb and from our temporal studies in our mouse model of hind limb ischaemia (HLI).

3. An optimal delivery strategy (good retention and activity/viability over defined delivery frequencies) for the administration of factors and/or cells at the sites of delivery in the ischaemic limb.

4. A molecular and/or cellular therapy ready for clinical trials.

5. A series of high impact publications that will disseminate our findings to the greater scientific and medical community and stimulate further work in this area.

Who or what will benefit from these outputs, and how?

Patients with limb ischaemia would not be expected to benefit from these outputs in the short term, as properly designed clinical trials would need to be instigated for any potential therapy to enter the clinic.

How will you look to maximise the outputs of this work?

We will closely collaborate with clinical colleagues to ensure that any therapeutic strategy derived from our work can be tested in clinical trials for safety and efficacy and ultimately be commercialised to ensure widespread clinical use.

We will also disseminate the results of this work at conferences and workshops and publish in peer reviewed national and internal scientific journals. Workshops will be

organised within our institution where we will share our results and provide hands on training for internal and external academics interested in this work. Other means of dissemination will include talks at the Pint of Science initiative or active participation in the summer festival of the Royal Society.

Species and numbers of animals expected to be used

- Mice: 7100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mouse models of compromised blood flow to the limb (ischaemia) will be used in this project licence because their molecular and cellular responses to ischaemia is similar to that seen in patients with this condition.

Typically, what will be done to an animal used in your project?

A. Mice used for the hind limb ischaemia model may undergo the following.

1) (i) Bred to express certain gene defects that will allow us to interrogate the importance of those genes in blood vessel development in legs with poor blood supply, or be given a diet that promotes a similar condition (fatty arteries) to that seen in patients with this condition.

AND/OR

(ii) Be given a diet that promotes a similar condition to that seen in patients with arteries that are narrowed because of fat deposits in their wall (atherosclerosis), and/or treated with a chemical to induce diabetes. This combined condition closely mimics Type 2 diabetes in man.

2) Undergo an operation to tie off or remove a section of a major blood vessels in order to cause a significant reduction in the blood supply to a rear limb (hind limb ischaemia, HLI).

3) Treatment with cells or agents that might help blood vessels to develop. These will be administered via direct injection into the mouse's ischaemic hind limb, injection via a blood vessel or infused by a mini-pump placed inside the mouse.

4) Imaged over time to see how well those blood vessels have developed and whether any cells that have been given are part of the developed blood vessels.

5) Have small blood samples taken at the time of imaging.

6) Humanely killed and blood and limb muscle tissue removed for analysis in the laboratory.

The duration of this experiment will typically be no longer than 21-28days

B. Mice used as a cell donors may undergo the following.

1) Bred to express specific gene defects so that cells obtained from these mice also express the same gene defect.

2) Administration of an agent to induce the production of inflammatory cells, via injection of the agent into their abdomen that will induce the production of inflammatory cells we believe may be important in blood vessel development.

OR

Administration of an agent via a mini-pump placed surgically into the abdomen that delivers the agent over a period of time to promote the release of cells from the bone marrow that we believe may be important in blood vessel development.

3) Humanely killed and cells removed from the abdomen or bone marrow for use in our mouse model of hindlimb ischaemia or analysis in the laboratory.

The duration of this experiment will typically be no longer than 5-14days.

C. Mice used for screening of agents or cells that have the potential to stimulate blood vessel growth may undergo the following.

1) Bred to express certain gene defects

2) Administration of a protein matrix plug containing cells or agents of interest via injection or small surgical incisions in the back of the mouse (maximum 4 places).

3) Imaging of the growth of vessels in the administered matrix plugs at defined intervals.

4) Humane killing and removal of the plugs for analysis of blood vessel growth in the laboratory.

The duration of this experiment will be no longer than 42days

All protocols have been refined so that the minimum experimental duration and animal numbers are used to achieve the scientific objectives.

What are the expected impacts and/or adverse effects for the animals during your project?

The mouse hindlimb (HLI) model is commonly used for the study of limb ischaemia as this condition is reasonably well tolerated by the animal, because there is an inherent recovery

of blood vessel function over a relatively short period of time. Animals may experience temporary lameness in the affected limb (up to 1wk), while the new blood supply is established. The protocol for this procedure has been classified as 'moderate' as a surgical procedure is involved and in a smaller number of animals (<10% of the number of animals) tissues of the toes in the affected limb may die (develop dry gangrene) and these toes break off naturally. Neither the lameness nor loss of digits appears to affect their well-being as the animals are seen moving around the cage, grooming and feeding and drinking normally. This is similar to patients with limb ischaemia, who may also experience loss of multiple digits and yet are able to mobilise independently without discomfort. Pain following surgery will be minimised by treatment with an appropriate analgesic.

The use of mice in which specific genes have been removed or manipulated to be switched on or off, and agents that induce conditions that are associated with increased artery narrowing leading to poor blood supply to the leg (e.g. atherosclerosis or diabetes) are also commonly used in investigations of the regulation of blood vessel development. The genetic manipulations and agents used to promote the build-up of fatty deposits in arteries may result in a small number of mice suffering adverse effects such as skin irritation that can lead to skin ulcers. Agents used to promote diabetes could result in higher blood sugar levels than is warranted and these can lead to infection and loss of life. Both of these conditions can be treated early with creams for the skin and insulin injections used to control levels of blood sugar (in much the same way we treat diabetics). Those animals that do not respond within a few days of treatment will be humanely killed.

Bone marrow transplantation between mice with different genes enhances our understanding of the importance of those genes on reparative processes in the ischaemic limb. While mice tolerate ablation of their bone marrow by irradiation reasonably well, it exposes them to the possibility of infection (immunocompromised). For this reason, irradiated mice are housed in independent ventilated cages during the time they are immunocompromised (up to 21days). Prophylactic measures (e.g. treatment with antibiotics) will also be undertaken to prevent the risk of infection. The mice also tend to lose some weight after irradiation, but most will recover the lost weight within a few days.

Mice used as cell donors may suffer adverse effects such as the build-up of fluid in the abdomen leading to abdominal tenderness on handling. Mice displaying this condition will be immediately humanely killed, and the cells that have accumulated in the abdomen removed for our investigations. Surgical wounds may rarely become infected or the stiches breakdown (<1% of cell donors) following surgery to implant a device (e.g. mini-pump) that can deliver an agent that promotes cell release from the bone marrow. These can be treated with antibiotics or resewing of the wound. If these treatments are not effective, the animals will be humanely killed.

Mice used in the screening of potential treatments that promote blood vessel development and undergo surgery for implantation of protein plugs may rarely (<1% of mice) experience, adverse events such as infection at the site of the implantation (by a surgical procedure), but this can readily be treated with an antibiotic.

All animals will be humanely killed at the end of an experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities and proportion of animals from all experimental protocols experiencing each severity includes:

1. Mild – ~ 74%

2. Moderate - ~ 26% (surgery carried out and in ~ 4% of these there may be tissue death leading to loss of toes)

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The processes of how blood vessels block and prevent oxygen reaching end target tissue such as muscle (ischaemia) and how the body may grow existing or new blood vessels to try and compensate for this, involve dynamic interactions between a myriad of molecules and cells that are impossible to replicate on the laboratory bench (in vitro). It is not ethical to study the effect of injecting substances and cells into patients with disease of their blood vessels without preliminary evidence that this form of intervention may be of benefit. We therefore have to carry out our initial experiments in an animal model of hind limb ischaemia (HLI), which allows the study of the dynamic cellular and molecular responses and interactions that take place during blood vessel growth in low oxygen environments, and assessment of the effects that treatments, derived from our studies in patients, might have on this process.

I am satisfied that the potential gain, in terms of treatment of human disease, is sufficient to justify the use of animal models.

Which non-animal alternatives did you consider for use in this project?

We use methods that employ cells cultured in the laboratory to assess mechanisms of how blood vessels grow and cellular or molecular interventions that might stimulate this process. We also use these as an initial screening tool when assessing which cells or molecules have the potential to promote robust blood vessel development in before they are used in our mouse models of hindlimb ischaemia. We continue to develop more complex cell culture systems that more closely mimic tissue compartments (organoids) to help us to interrogate pathways that may be involved in blood vessel development in ischaemic environments and for use as extra screening tools prior to the use of our mouse models.

Why were they not suitable?

Studies in the laboratory cannot truly replicate the complex, dynamic interactions that take place between the many factors and cells that are involved in the development of an existing blood vessel or the formation of a new blood vessel.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals required for our experimental studies based on our previous experience with the HLI animal models and of breeding for use in these models. Of the total number of animals we expect to use over the 5yrs of this project

- ~70% of animals will be used in breeding and maintenance (Protocol 1)
- ~21% will be used in for the hind limb ischaemia experiments (Protocol 2)
- ~4.5% as cell donors (Protocols 3&4)
- ~4.5% for the in vivo neovascularisation assays (Protocol 5)

We have estimated the number of animals required for our experimental studies based on our previous experience of the variability in the end-point measures (e.g. degree of improvement in blood perfusion, number of vessels and size of vessels) in these models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In the HLI model we use the unaffected leg as an in-built internal control against which we can assess the recovery in blood perfusion (perfusion index) in the ischaemic limb in the

same animal over time. Our extensive experience with these models in a variety of wild-type and genetically modified strains of mice has provided us with a good analysis of the variability in the endpoint measures that we have developed with these models, and we have used this experience to carry out appropriate statistical power calculations for the numbers of animals required in our experiments.

Where we find ourselves using a strain of animal or treatment that we are unfamiliar with, or there is little data in the literature on, then we will carry out pilot experiments using longitudinal or dose escalation analysis in small cohorts of animals in order to provide statistical data that allows animal number estimations for definitive larger experiments.

Other measures we have taken to reduce animal numbers includes the development of 'test tube'- based analytical methods systems for the laboratory screening of cells or agents highlighted by our human studies as potential modulators of revascularisation. We will also use a simplified in-animal screening model that allows assessment of multiple cells or agents, or doses of these, in the same animal, prior to their assessment in our more complex models in which we induce limb ischaemia. We will also use imaging techniques (such as laser Doppler blood perfusion imaging (LDPI) that allow measurement of limb blood perfusion in the same animal over time (longitudinal imaging), thereby reducing the numbers of animals required for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will always carry out pilot studies using small cohorts of animals prior to embarking upon a new experimental plan. If we are planning to interrogate ischaemic tissue, we will first check repositories of tissue banks that may be available to use, prior to obtaining our own specimens. We also work with other scientists who are international experts in their field and use the hindlimb ischaemia model. We will discuss our experimental strategy with them to optimise the number of animals we use and determine whether tissues and data can be shared between our groups.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Species: Mice are the lowest mammalian group in which models of hind limb ischaemia have been successfully characterised and are therefore the species most often used in the

study of limb ischaemia. In our experience mice tolerate the ischaemia reasonably well, as new blood vessels develop spontaneously in the affected limb through processes similar to those seen in man. The mouse models have the advantage that they also allow the use of animals in which we can test the activity of specific cells of human origin without rejection; as well as facilitating investigation of the importance of specific genes on blood vessel growth through genetic modification in these species.

Animal models: Mice tolerate surgery well, with good recovery rate and low post-operative infection, but as a result of the reduced blood flow, animals may have temporary lameness for a few days and a small proportion may have toe loss in the affected limb (in much the same way as we see in patients with critically ischaemic limbs). Existing blood vessels (collaterals) increase in their size and, together with new blood vessel growth, establish blood flow. The lameness (or loss of toes in some) does not appear to affect their well-being as they are seen moving around the cage and feeding, drinking and grooming normally within 24hrs of the operation and for the remainder of the experiment's time course (up to 42 days).

The techniques to evaluate blood vessel growth in this model such as angiography (X-ray based imaging of blood vessels), laser Doppler perfusion imaging (LDPI) and analysis of muscle by specific staining of blood vessels in sections of muscle (histology), are well characterised and internationally accepted in these models.

Why can't you use animals that are less sentient?

Less sentient animals cannot be used as they do not have a vasculature that replicates that of man or allow interrogation of the complex processes giving rise to blood vessel development in the context of ischaemia. Mouse models also have the advantage that they allow testing of the activity of specific cells of human origin without rejection.

Blood vessel development needs to be assessed over a period of time that precludes assessment under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Pain relief is given during and after the operation, to minimise suffering, in much the same way that we treat patients. Animals will be monitored daily at early stages post-surgical interventions and at least 3 times weekly once early clinical signs have developed.

Inhalation anaesthesia will be used wherever possible to minimise transient pain and distress, e.g. during surgery and imaging. In addition, full recovery between periods of anaesthesia, rehydration during long imaging sessions, respiration/cardiac function monitoring, body temperature monitoring/maintenance will be conducive to animal wellbeing.

In animals in which diabetes is induced, we will monitor glucose levels and treat mice who develop very high blood glucose levels (severe hyperglycaemia) with slow release insulin to bring this down to an acceptable hyperglycaemia.

Animals to be exercised will receive up to 5min of training on 2 consecutive days prior to their inclusion in full experiment on effect of treatments on distances covered following HLI. Those that do not respond to the exercise regimen will be removed from the exercise component of the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow established published guidelines to ensure experiments are conducted in the most refined way. These includes:

1) The Responsibility in the Use of Animals in Bioscience Research guidlines produced by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

2) The Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

3) Accepted limits of volumes and frequencies when administrating compounds and anaesthesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed by updates from the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) website and seminars on the 3Rs organised within and outside of our institution.

We will also have direct support and contact with an NC3R’s regional Programme Manager who supports the application of the 3Rs in our and other major institutions and is a member of the Policy and Outreach Group. This includes providing expert advice and coordinating the sharing of best practice.

42. Visual processing in frogs

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Vision, Computation, Retina

Animal types	Life stages
Xenopus laevis	adult, embryo, neonate, juvenile
Xenopus tropicalis	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We want to better understand how neural circuits in the retina and brain process visual information, and how these circuits evolve.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We are pursuing this project because relatively little is known about the diversity of mechanisms by which neurons transmit different kinds of sensory information. These mechanisms play a key role in determining what we can and cannot see, and we need to understand these processes as they operate in the intact retinal circuit.

This project will advance science in several ways. First, we will improve our understanding of the processes which transfer signals between nerve cells. Defects in these processes

have been associated with important diseases, such as Alzheimer's and Parkinson's. Second, we will improve our understanding of the variety of ways in which visual signals are processed in the brain.

What outputs do you think you will see at the end of this project?

We expect to obtain information about signal processing in the visual system, that we will communicate through scientific publications.

Who or what will benefit from these outputs, and how?

The most immediate beneficiary of our research will be the scientific community devoted to vision research. In the mid- and long-term, our findings could have the potential to be incorporated into the frameworks of ophthalmological and neurological research for clinical applications, and/or in those of ecological and evolutionary research for conservation efforts of species that live under different illumination conditions.

How will you look to maximise the outputs of this work?

We will collaborate with other research groups across the world that work on related topics, and exchange findings, new tools and methods develped, etc. in events such as conferences and seminars, to ensure that progress is made as steadily as possible for everyone involved. Furthermore, details on any new tool or method developed during the project will be made widely available in open source repositories.

Species and numbers of animals expected to be used

- Xenopus laevis: 50 adults + 10000 tadpoles
- Xenopus tropicalis: 50 adults + 10000 tadpoles

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Frogs are a key step in the evolutionary transition of vertebrates from water to land. Moreover, frogs lack "key higher brain regions", such as a mammalian-like cortex, and thus represent a good trade-off for studying fundamental principles of vertebrate vision with fewer ethical implications than e.g., mammals.

We will use both adults and tadpoles for behavioural experiments. Adults are needed as the tests have been developed and standardised for that life stage, and we need those data to confirm previous results on other types of frogs. However, for physiological work we will use tadpoles, because they are transparent and allow us to run our experiments

using non-invasive recording techniques. To then link these two sets of experiments, we will also perform behavioural work on tadpoles.

Typically, what will be done to an animal used in your project?

Adult frogs and tadpoles will be used for non-invasive behavioural experiments in which they will be allowed to follow their innate preferences for visual stimuli. Some frogs will also undergo a harmless injection with hormones to obtain eggs (breeding).

Tadpoles will be obtained from wild type or genetically manipulated eggs and sperm, and they will typically be anesthetized and/or paralysed with appropriate doses of tried and tested drugs to keep them immobile. While in this condition, we will show them different visual stimuli while we observe them under a two-photon microscope to record the activity of neurons in response to those stimuli. Each of these experiments will last for a maximum of 3 hours, and the vital signs of the animals will be monitored throughout. Tadpoles will be humanely killed immediately after each recording session.

What are the expected impacts and/or adverse effects for the animals during your project?

None of the procedures that we will apply is expected to generate adverse affects, as they are non- invasive, done under anesthesia, or both. Some of the transgenic tadpoles that we will use might turn out visually defective or blind, and in such case they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity for procedures involving adult animals is mild or subthreshold in all cases. For tadpoles, up to 50% of the animals might undergo procedures classified as moderate.

What will happen to animals at the end of this project?

Used in other projects
Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Behavioural experiments can only be performed in live animals, and they are necessary for confirmation of the sensory abilities enabled by the visual system of Xenopus.

For our in vivo physiological work, we have no alternative but to use the intact retinal circuit or the whole tadpole brain. Using the actual, unperturbed, neural circuit is the essence of our approach, as there is no substitute for the retina or brain to understand how each of them work.

Which non-animal alternatives did you consider for use in this project?

Retinal organoids are the most sophisticated tool available at the moment, and they can provide valuable information about some aspects of retinal cell biology.

Why were they not suitable?

Organoids remain at best incompletely explored for the purpose of understanding visual function in the intact circuit. Retinal organoid technology in general is not yet at a stage where it can realistically replace in-vivo or ex-vivo preparations. Moreover, organoids are mostly being explored in the context of mammalian vision - to our knowledge retinal organoids of anuran origin are non-existent.

Moreover, retinal organoids cannot "see" and are not connected to the brain in the way the visual system is in the intact animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For breeding and stock replacement, we estimate that we will need no more than 50 animals over the five year project duration. Of these, ~20 will also be used for adult behavioural experiments

For the tadpoles, we estimate that we will need a total of approximately 10,000 individuals to collect enough physiology and behavioural data from a variety of stimuli from an appropriate number of replicates, and taking into account that expression differences between animals will inevitably lead to the need of a surplus in breeding.

However, the vast majority of embryos (~90%) will not reach the developmental stages that we will use for the actual imaging/behavioural experiments, but instead only be grown until expression of desired transgenes (e.g. GCaMP variants) can be non-invasively acertained (e.g. fluorescence screening). An expected large fraction of non-expressing

animals, as well as any potential surplus in well-expressing animals for immediate experimental requirements, will be humanely killed. Moreover, we also take into account the fact spawns contain many eggs, and that sometimes not all of them will reach the developmental stage that we need.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All visual stimuli that we will use in behavioural and physiological experiments are designed to be harmless, meaning that each individual animal can be repeatedly tested for many stimuli, so we will use substantially fewer animals than we would need if each animal would be exposed to just one single stimulus. These repeated measures will also allow to reduce variability and enhance statistical robustness while keeping the number of animals to a minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As both the behavioural experiment and the hormonal induction of ovulation are mild (or subthreshold), non-invasive procedures, the same females from which we will obtain eggs will be also used for the behavioural tests.

Furthermore, whenever an adult animal needs to be humanely killed, their retinas can be used for ex- vivo experiments and/or preserved and processed for other studies, avoiding the need to use more animals specifically for these two purposes. These additional use-scenarios are all ongoing in the lab.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use frogs, which are thought to be amongst the "least sentient" representatives among tetrapods. We will use adults only for behavioural tests that do not involve administration of any substance, and for obtaining eggs through mild, standardised and safe procedures.

All physiological work will be done on more immature life stages (tadpoles), using established harmless stimulation and recording procedures. Immobilisation of the animals

will be accomplished using anaesthesia and embedding in low-melting point agarose, and the general wellbeing and clinical status of the animals will be monitored at all times.

Why can’t you use animals that are less sentient?

Frogs are amongst the least sentient tetrapods, and our physiological work will be done on their most immature (tadpole) stage.

Terminal anaesthesia for physiological imaging experiments will only be avoided in those cases in which we need to confirm that the data obtained under anaesthesia really represents what happens in the unperturbed visual system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our procedures are designed in such a way that they minimise the welfare cost to animals by relying on non-invasive techniques that also allow to control for markers of stress levels and overall clinical status in real time during the procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will rely on the Animal Scientific Procedures Act for general guidance, and on Xenopus-specific guidelines such as the Animal Care Protocols form the Marine Biological Laboratory at University of Chicago (https://www.mbl.edu/xenopus/protocols) and Guidance on the housing and care of the African clawed frog Xenopus laevis from the Royal Society for the Prevention of Cruelty to Animals (https://www.rspca.org.uk/webContent/staticImages/Downloads/GuidanceXenopusLaevisReport.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our main source of general information about advances in the 3Rs will be the newsletter from the National Centre for the Replacement Refinement & Reduction of Animals in Research (NC3Rs), which will keep us updated about novel approaches and developments and training opportunities. For advances specifically related to our experimental animals we will be in close contact with the European Xenopus Research Centre to learn about any potential advances in Xenopus-related protocols that could be directly applicable to our project.

43. Production of normal blood

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Assay, Red Blood Cell, Antigenicity, Diagnosis, Surveillance

Animal types	Life stages
Ferrets	adult
Guinea pigs	adult
Turkeys	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will be used to provide normal blood that will be used in in-vitro diagnostic or research assays relating to microbial diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

- It is important to carry out this work because the microorganisms that cause disease in humans and animals can be demonstrated in the laboratory using specific animal blood and tissues, for example, the way viruses have the ability to attach themselves to the host can be illustrated by mixing virus particles with mammalian/avian red blood

cells which have specific pathogen receptors on their surface. We also carry out tests to ascertain the likelihood of an immune response of influenza/viral pathogens, mammalian and avian red blood cells are vital for these tests.

What outputs do you think you will see at the end of this project?

- The outputs will be data and knowledge used for the diagnosis, control, or prevention of communicable diseases that primarily affect humans but could have originated in animals.

Who or what will benefit from these outputs, and how?

- The benefit from this project is great as far as diagnosing new and re-emerging strains of influenza, the work also contributes to the UK vaccine development which leads to positive outcomes in terms of the benefits to public health. There are a network of surgeries that send samples of patients with suspected influenza to be tested, they rely on the diagnostic services which acts as a surveillance tool that helps forecast what strains of the disease is circulating. With this information interventions can be made i.e. vaccine development , pandemic curtailment. This is a direct public health benefit.

How will you look to maximise the outputs of this work?

Global network participation, presentations and publications are routine mechanisms. These publications inform both national and international agencies insiders and the public of the progress and benefits of our work such as surveillance of influenza and other respiratory viruses in the UK which will be shared via digital platforms.

Species and numbers of animals expected to be used

- Other birds:
- No answer provided
- Guinea pigs: 400
- Ferrets: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals that are used in this project are the most appropriate species for the purpose. For example, we use turkey (avian) and guinea pig (mammalian) red blood cells as they are highly compatible with influenza. This allows us to differentiate between different strains of influenza and therefore they are a vital part of the identification and control of the disease.

It is more advantageous to use young adults rather than infants or older animals as young adults are at the peak of their physiology and less likely to suffer from age-related illnesses or organ immaturity and this is a possibility if immature or aged animals are used. The blood and tissues are therefore optimal for the in-vitro assays

Typically, what will be done to an animal used in your project?

- Invariably general anaesthesia is used briefly to obtain samples of blood from the animals as this reduces handling stress, makes for easier handling and therefore eliminates contingent (albeit minimal) distress/suffering. Where general anaesthesia cannot be used where possible we will opt for a local anaesthetic instead to minimise the harms.
- The red blood cells will be collected and used for diagnosis, surveillance, control and prevention of communicable diseases.

What are the expected impacts and/or adverse effects for the animals during your project?

- The impact on the animals during this project is mild, any pain, suffering, and distress will be transient. We do not expect any animals to exceed the prospective severity of the protocols.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- The severities of this project are non-recovery and mild, any pain, suffering, and distress experienced will be transient. The turkey and ferret protocols are mild, and all guinea pig procedures are non-recovery.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

- We currently use animal red blood cells in a laboratory setting for testing that supports the diagnosis and surveillance of communicable diseases.

- Synthetic blood products are not available commercially or within the scientific community; the work we carry out would not be possible without the use of red blood cells. We cannot use a lower species such as fish or mice for example, as their red blood cells are not compatible with influenza viruses (new, emerging and re-emerging) that affect humans, also they are too small to produce the volumes of blood needed.

Which non-animal alternatives did you consider for use in this project?

- There are no suitable non-animal alternatives for the tests that we carry out.

Why were they not suitable?

- Some specific mammalian and avian red blood cells have a protein coating that binds to specific differentiating influenza antigens. At present, there are no suitable methods that achieve the same results as the tests that are agreed as the 'gold standard' and standardised across the world.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

- These numbers are based upon the experience of 40 years of using the techniques to determine animals to be used in a ‘normal’ non-pandemic or routine use scenario plus additional that would be used to consider a spike in infections across the UK.

- These numbers provide a surge capacity in the case of an epidemic/pandemic that enables us to provide a critical service in the prevention and control of transmissible diseases.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- There is no experimental design phase to the process.

- The red blood cells are used routinely ex-vivo and in-vitro, i.e. the testing is carried out on the blood samples collected from live animals and used in the laboratory.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Animals are procured at the point of need in order to keep the numbers to a minimum and animals can be re-used on this and other projects once they have passed a health assessment carried out by the NVS.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Ferrets, turkeys, and guinea pigs will be used in this project. Ferret, guinea pigs, and turkey red blood cells are used for specific tests, all the red blood cells mentioned react differently to different strains of influenza e.g. avian RBCs respond well to H1N1 and guinea pig responds well to H3 subtypes. To minimise pain, suffering, distress or lasting harm blood samples are invariably taken under general anaesthesia.

- Non-recovery procedures are always conducted under general anaesthetic. All the procedures are brief.

- Where blood is withdrawn other than no-recovery, samples amount are limited e.g. birds will have no more than 10% taken at one time or 15% in a 4 week period.

- Ferrets will have no more than 10 % taken at one time. We always take less blood than the volume percentage mentioned as this supports good welfare.

Why can’t you use animals that are less sentient?

- We must consider the maturity of the cardiovascular system to be able to produce viable red blood cells. Immature animals are not able to produce the volumes needed.

- Guinea pigs are terminally anaesthetised in order to collect large volumes of blood from the heart, this is the most humane method to collect large volumes of blood from this species in a single event.

- Anaesthetic is used to significantly minimise any pain and suffering when collecting blood from the superficial veins of turkeys and ferrets.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- The procedures are already well refined from experience over many years of their use. We always aim to minimise pain, suffering, distress, or lasting harm.

- We use the most appropriate methods for collecting blood samples and use anaesthesia where the use of anaesthesia will cause less harm than the procedure itself.

- Post-procedure observations are carried out and the animals are monitored throughout the project e.g. via strict weight monitoring and health assessments.

- Turkeys and ferrets enjoy human interaction which they receive daily. All animals are housed in floor pens and have plenty of environmental enrichment which keeps them mentally stimulated.

- All animals are fasted or have food withdrawn 20-30 minutes prior to undergoing a procedure involving general anaesthetic. Guinea pigs are physically incapable of vomiting but they can regurgitate, hold food in their oesophagus and buccal cavity that can be aspirated into the lungs when under general anaesthesia. This is eliminated by withholding food prior to the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

- We follow best practice regarding experimental techniques such as blood-letting from literature such as The Manual of Animal Technology by Stephen W. Barnett, other reliable online resources such as Understanding Animal Research 'Procedures with Care': a training resource on the administration of substances and the Handbook of Laboratory Animal Management and Welfare by Sarah Wolfensohn.

- The above literature gives detailed guidance on animal management, welfare, and the correct/most refined methods of experimental techniques.

- The experimental techniques used under this project only consist of blood withdrawal that is always of mild or non-recovery severity.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- I am connected to the NC3Rs i.e. we are signed up and receive newsletters informing us about advances in animal science. I am looking to advance the refinement through environmental enrichment programmes and all the animals used in this project are floor housed.

- I am an associate member of the Institute of Animal Technology and receive regular correspondence.

- I stay informed via discussion with the NVS and through networking with colleagues from other organisations.

44. Breeding and maintenance of genetically altered and germ free mice

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Rederivation, Germ Free, Cryopreservation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To provide genetically altered and germ free mice to the researchers in the establishment. This project also attempts to cryopreserve lines with no immediate use, and rederive clean colonies from animals with lower heath status.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The main benefit of this project is that animals required under the authority of various project licences, will be bred centrally under the best possible conditions. This will allow management by trained animal technologists who have expertise in the breeding and colony management, ensuring the husbandry of these animals will be in full control of the breeding programmes. In addition, a centralised facility for breeding ensures that the

technicians, by carrying out the work as a routine, are well skilled in the techniques involved.

Under this licence, the number of animals bred will also be regularly reviewed through discussions with the end users and animals will only be bred if a requirement has been established and the authority for the use of such models have been granted to the end uers. New strains or models may be imported onto this PPL as long as authority exists to use such a model on the end users' project.

Some strains for importation may only be available as embryos, typically cryopreserved, or may be of an unacceptable health status, in which case we will need to import them into our facility via embryo transfer.

In addition, despite the maintenance and management of high barrier animal housing facilities, our mice at some point may become exposed to pathogens, which can adversely affect their welfare or interfere with experimental results. This project licence seeks to use well-established embryo transfer techniques to rederive clean, previously pathogens-exposed, or to produce germ free mice. This will benefit our research community. Furthermore, the use of microbiologically defined animals kept under good husbandry conditions will reduce the number of animals required for each study, while the absence of disease can be considered a refinement in the animal's life.

Since, novel genetically altered strains are continually being produced and new research models being created the need for a service licence to support the basic and transnational research of the scientific groups at the institute is likely to continue.

What outputs do you think you will see at the end of this project?

This licence will be used to import animals ahead of new research groups arriving at the university. This will facilitate the research of these groups by providing cohorts of animals as soon as they have Home Office approval.

In order to support the 3Rs within the scientific community, there has been increased demand for research groups to cryopreserve mouse embryos of strains which are currently not in use, with a view to subsequent re-implantation at a later stage when the requirement for use is established. The cryopreservation procedure will be carried out under this licence.

In addition, on site rederivation will enable us to clean up imported animals with unacceptable health status, before introducing them into our SPF(Specific Pathogen free) facility. This not only benefits the animals' general wellbeing but also addresses any issues concerning animal welfare, reducing the variability in experimental results as animals are free from defined pathogens.

Breeding germ free mice will assist the users to investigate the effect of intestinal microbes in immune cells cross-talk and to determine how specific microbiomes protect from or contribute to disease.

Who or what will benefit from these outputs, and how?

The research community will benefit as we will produce good quality animals for their research with accurate breeding and phenotyping data. By producing animals which have good genetic integrity the reproducibility of experiments is improved. By using colony calculations we can reduce the number of animals being produced and potentially wasted.

How will you look to maximise the outputs of this work?

We will have animals which are good quality in terms of genetics as we will follow best practice. This involves refreshing the background of strains by mating them out to the genetic background on regular basis.

Strains with either mixed or ambiguous background strain information will undergo genetic monitoring to confirm the background substrain. By carringing out gentic monitoring it will give confidence to the reproducibility of the work.

We also use the breeding data which is generated from GAA colonies maintained under the service licence in colony sizing calculations. Using sizing calculations will help to make sure that the colones are sized to produce the number of animals required for experiments whilst also not overproducing animals which will be killed as surplus.

Breeding and maintaining animals under one service licence can reduce the number of mice which are potentially wasted. The use of service licence is disseminated through our animal users and animal wefare meetings, and the benefits of it in reducing the number of animals wasted, genetic integrity and reproducibility will be discussed with the researchers.

Species and numbers of animals expected to be used

- Mice: 23,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to fully understand the effects of human genes and their disease-associated mutants there is no suitable substitute for a mammalian model. The complex interaction between molecules, in the majority of cases, cannot be replicated in culture and in-vitro assays cannot adequately model the complete array of cellular, physiological and

behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes. We will be using animals of all ages and stages of development in the breeding protocols.

For crypreservation, we will use females between the age of 3-4 weeks old as it has been shown that females in this age bracket produce a higher yield of ova and a higher percentage of females which are mated produce fertilized ova.

The germ free mice are used to investigate the effect of intestinal microbes in immune cells cross talk and to determine how specific microbiomes protect from or contribute to disease. The colonisations and investigation on the effect of microbiome will be carried out under end-uers' PPL.

Typically, what will be done to an animal used in your project?

Most animals will be bred and maintained with no welfare issues. They will be maintained up to 15 months of age. Where required animals will be earmarked to obtain a sample for genotyping. Females may be injected twice by hormones 46-48 hours apart to generate transgenic embryos for the cryopreservation of lines or rederivation. A very small number of males may undergo surgery for vasectomy and will experience short-lived post-operative pain and discomfort. Embryos and blastocysts will be implanted surgically under inhalation anaesthesia or non-surgically into the reproductive tract of a mouse rendered pseudo-pregnant by mating with a sterile male.

What are the expected impacts and/or adverse effects for the animals during your project?

There might be adverse effects due to genetic modification of the genome. Information on expected phenotypes will be collected prior to breeding and will be recorded in a colony passport. Any animals on mild breeding protocol exhibiting any unexpected harmful phenotypes or deviation from normal health will be killed, or will be transferred to a moderate protocol of a PPL with the relevant authority on which the harms can be suitably monitored. Intraperitoneal or subcutanous injections should involve only slight and transient pain. Animals undergoing surgery for vasectomy or implantation of embryos will only experience short-lived post operative pain and discomfort. Ear notching should involve only slight and transient pain and no healing problems. If blood sampling is taken, then any bleeding will be controlled by local pressure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the mice on breeding and maintenance protocols we expect the majority to experience subthreshold severity (approximately 85%), and a small number to experience mild severity

(approximately 5%) and less than 10% to suffer moderate severity.

With the superovulation procedure we expect 100% of mice experience mild severity.

Animals undergoing surgical embryo transfer or vasectomy will experience moderate short-lived post- operative pain and discomfort (100%).

Animals undergoing non-surgical embryo transfer will experience no more than mild transient discomfort and no lasting harm.

What will happen to animals at the end of this project?

Used in other projects
Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice are required to supply the needs of the end users' project licences. In order to fully understand the effects of human genes and their disease-associated mutants there is no suitable substitute for a mammalian model. The complex interaction between molecules, in the majority of cases, cannot be replicated in culture and in-vitro assays cannot adequately model the complete array of cellular, physiological and behavioural interactions necessary to fully understand how genetic modifications result in normal or abnormal processes. The justification for their use lies with the end users' project licences and cell culture, human tissue assays will be used whenever possible.

Which non-animal alternatives did you consider for use in this project?

The aim of the service licence is to provide end users with mice for their studies and the consideration of non-animal alternatives will depend on the end users' area of research. Any researcher who wishes to use this service licence is requested to provide justification of why they wish to use this licence and the details of any non-animal alternatives they have considered. They will also be requested to provide the detail on whether they have explored and used search engines to find non-animal alternatives.

Why were they not suitable?

This licence aims to provide end users with mice for their studies and will only be done when non animal alternatives have been considered and rejected. The reasons for this will vary between projects but typically the reasons are that a whole organism is required to investigate the interactions between cells or organs in the body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have extensive experience of calculating animal numbers on a previous licence and plan to carry on with this practice. I work with end- users to calculate the minimum number required to reach their scientific goals. This involves estimating the final number of animals required and then calculating the number of breeding animals required using existing best practice guidance. This includes ensuring the appropriate age of animals used for breeding, replacement of breeding animals before productivity declines and the use of production efficiency index calculations to size the colony. All colonies on the service licence undergo a regular review where the productivity of each breeding pair/trio is assessed and any which fall outside of our guidance is removed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Animals will be bred if a user requirement has been established. The user will be expected to provide a rationale for the number of animals required to meet their scientific goal. We will calculate the number of breeding animals required using known breeding data. The breeding programme will be subject to regular review to meet anticipated demands while avoiding overproduction.

Strains that are no longer required will be cryopreserved which will preserve the ability to revive the model should it be required but without the need for continued breeding. The use of superovulation significantly increases the numbers of embryos which can be harvested from each mouse and therefore means that fewer animals are required overall.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding animals under a service PPL provides the certainty that animals are bred only to requirement. The strains will be overseen by experienced animal technicians to ensure that the breeding programmes designed for the colonies are followed. This will help to minimise any potential wastage from overbreeding.

This service licence also provides the opportunity to consolidate the breeding of models used by several end users. This would prevent the wasteful maintenance of several smaller separate colonies to provide each of the end-users with these animals.

Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs, and breeding colonies monitored closely to avoid inefficient and non-productive crosses. Samples for genotyping will be taken in timely manner so that the incorrect genotypes are not kept within the colony.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used because many transgenic and knockout mutant mouse strains have been created as disease models. The models that are bred on this project are typically used for experiments to investigate basic mechanisms that regulate activation of immune cells. Germ free mice are used to investigate the effect of intestinal microbes in immune cross-talk and to determined how specific microbiomes protect from or contribute to disease. By consolidating the breeding of various lines we can ensure that breeding and maintenance is done in the most refined manner. This includes efficient breeding practices and the calculations used to size the colonies. We also routinely refresh inbred lines to ensure the genetic integrity of the lines and prevent potentially harmful genetic drift.

Where a line is no longer being actively used this would be cryopreserved which involves superovulation. This is performed using a very established procedure and is considered to be the most refined. In brief, this involves two timed intraperitoneal injections prior to breeding. This procedure will allow the production of oocytes for in-vitro fertilisation, the production of fertilized embryos or the generation of blastocystes from mated superovulation. Vasectomy will produce males with full mating behaviour but are infertile.These males will be used for mating with females to produce a pseudo- pregnant state. The females are used to provide foster mothers for embryos revived from frozen storage or for rederivation purposes. Inhalation anaesthesia will be used during the aseptic surgery and analgesia will be provided pre-operatively.

Why can’t you use animals that are less sentient?

The justification for a particular line of mice lies with the end users’ Project Licence. Typically, the justification for using is that lower-order organisms do not exhibit the

required features. Mice are required for work involving adaptive immune response or investigations into ageing diseases.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Best practice will be applied when designing breeding strategies to ensure genetic stability. Project licence holders will also be advised and encouraged to routinely cryopreserve their lines for both disaster recovery and genetic stability reasons. Where substances are administered by injection the minimum effective dose and most refined route will be chosen.

Analgesia will be provided pre-operatively. General anaesthesia will be induced and maintained using agents and routes suitable for the species, nature and duration of the procedure. Surgery will be performed under aspetic technique.

We will also evaluate the new non-surgical methods for transferring mouse embryos to recipients. If these methods prove superior to surgical methods i.e. by refining the technique and reducing mouse numbers, they may be adopted as standard. Where possible, genetically sterile T145 mice will be used as non-surgical alternative for vasectomy.

Whilst this service licence includes the option for producing animals for cryopreservation by embryo we will always recommend sperm freezing, as the most refined method of cryopreservation, before we agree to carry out embryo cryopreservation.

All strains are bred in a fashion to avoid infection. Routine health checks are carried out to monitor welfare. The animals are bred by a dedicated team of trained staff with experience in the care of such animals. Environmental enrichment, including nesting material is provided to accommodate natural behaviour of the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office efficient breeding guidance will be followed as a routine practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Recommendations and updates regarding aspects of housing, husbandry and welfare of transgenic strains of mice will be consulted throughout the duration of this licence, and published guidelines for best practices will be followed. We will use effective breeding programmes for breeding of colonies in line with the principles of the 3R's. The information is also distributed through the establishment to other licence holders who maintain breeding colonies. These guidelines will be regularly reviewed based on any advancements made in this area. These advancements will be identified through regular

attendance of conferences (LASA, IAT Congress, NC3R's tech symposium, etc.) and comparisons to other large breeding institutions. We receive frequent updates from these institutions as well as information from the NC3R's, Norecopa and our internal 3R's subcommittee. The 3R's subcommittee collects advancements from across the establishment and disseminates this information. This enables the wider implementation of the advancements made by individual groups.

45. Molecular imaging and radionuclide therapy research for cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cancer, imaging, molecular radiotherapy, biomolecules, cellular therapies

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

One of the central aims of this project is to develop new radiopharmaceuticals for nuclear imaging (positron emission tomography (PET) and single photon emission computed tomography (SPECT)) and molecular radiotherapy of cancer. We will develop, evaluate and use non-invasive imaging tools, techniques and protocols in living animals to study the targeting, biodistribution, pharmacokinetics and/or pharmacodynamics of new imaging contrast agents, biological therapies, radionuclide therapies, or targeting vectors for cancer. We will also develop imaging tools that allow quantitative and non- invasive measurement of disease in rodents at different timepoints, thus reducing the numbers of animals needed to evaluate therapies or biological processes in cancer research.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Imaging is a vital tool for clinicians in managing patients with cancer, used in diagnosis, treatment planning, measuring response to therapy, surveillance and evaluation of recurrent disease. There is a great need to develop new, cancer and target specific imaging agents, able to give information not only about tumour location but also biochemical status and patient prognosis. A great many of these imaging agents are themselves biological molecules that bind to particular targets on the surface of cancer cells or are taken up by cancer cells in preference to normal cells thus making it easier to visualise disease. The likelihood of response to targeted therapies is increased if the tumours highly express the receptor of interest. Tumour receptor density can be evaluated via injection of radiolabelled, gamma-emitting versions of the targeting agent suitable for imaging. These targeting agents can also be radiolabelled with alpha- and beta-emitting radionuclides which can directly kill tumour cells in their vicinity and this is known as molecular radiotherapy. Currently there is great interest in evaluating new molecular radiotherapy agents with a range of isotopes, especially in combination with current treatments to which the tumours usually develop resistance. The hope is that tumour cells and chemo-resistant cells can be overcome using molecular radiotherapy, a systemic treatment which has a completely different mechanism of action and is effective against even small clusters of cells (known as micrometastases) which are too small to detect. Imaging is also vital for evaluating new therapies in vivo. Currently there is great interest in the development of cell therapies. For example, CAR T cell therapy- a type of immunotherapy- utilises T cells from patients, which have a critical role in the immune response by killing infected cells. T cells are initially isolated from blood samples of patients and modified in the laboratory to make them express special protein receptors on their surface, called CARs. These CARs recognise specific proteins, known as antigens, on the surface of cancer cells. The modified T cells, now known as CAR T cells, are then multiplied and infused back into the patient where they recognise, target and kill cancer cells expressing the target antigen. We would like to study these cells in living subjects in order to inform dosing, biodistribution, expansion and persistence. These cells can be directly radiolabelled and tracked in vivo for days/weeks or genetically modified to take up imaging probes and tracked in vivo over months. This information will speed up the development of these exciting new therapies and allow evaluation of efficacy and toxicity, vital for reducing the risk to patients. As well as providing benefit to patients, non-invasive imaging allows measurement of disease burden in experimental animals without culling. Computer models to do this more accurately and efficiently can help researchers to evaluate their new therapies in animals that have tumours deep within their body. This reduces the numbers of animals needed for experiments as well as reducing suffering through more accurate measurement of disease burden.

What outputs do you think you will see at the end of this project?

Our outputs will be new data, new radiotracers, new molecular radiotherapy agents and publications. We will generate pharmacokinetic, efficacy and dosimetry data and use them to apply for ethics approval and funding for clinical trials in the area of molecular imaging and therapy agents. This work will allow us to have as much information as possible to optimise the design of the clinical trial and to supply data to MHRA and ethics committees. Animal imaging studies will provide information on the likely effects of timings and combinations of different therapies on cells and what molecular changes are seen in the cells in response to the therapies. In molecular imaging and radiotherapy studies, animal studies are used to determine the radioactivity dose to the target tissues (as well as the normal organs) to determine efficacy and dosimetry for human studies. In some cases, the data obtained from imaging studies will be validated by reference against molecular studies on tissues samples taken for ex-vivo analysis. Targeting studies using radiolabelled antibodies, peptides, cells etc will generate data on target density, accessibility and predict how well the treatments will work. Our results will also be used to eliminate any imaging or therapy candidates that do not meet our requirements in terms of targeting properties, visualisation of disease or therapeutic efficacy with the minimum possible toxicity/off target effect.

This project will provide a set of building blocks, molecular tools and reagents for modifying cells, enabling the cells to take up radioactive molecules for imaging. We will also develop techniques and mathematical models to image and quantify cell therapies in vivo. Furthermore, the project will provide new computational machine learning tools and imaging tools for measuring the tumour microenvironment in living animals, thus allowing us to reduce and refine the numbers of animals used in scientific research.

Who or what will benefit from these outputs, and how?

The short term the beneficiaries will be ourselves and others in the scientific community (both academia and industry), to use the data produced to move the field forward or to use our tools to speed up development. In the medium term, the benefits are to bring new therapies/molecular radiotherapies to the clinic as well as new diagnostic imaging agents. Other scientists will be able to use the computational tools we are developing to obtain more (and better) in vivo data from each animal and reduce the numbers of animals being used in these experiments. The long term beneficiaries will be patients. We hope to have an impact on patient survival or to achieve improvements in patient management, be it from new molecular imaging and therapy agents, more effective combinations or targeted drugs, faster development of cell therapies for solid cancers and new therapies for pancreatic and other cancers that are based on modulation of the tumour microenvironment. These will be used by ourselves and others who are developing cell therapies.

How will you look to maximise the outputs of this work?

Our results will be disseminated internally and within our collaborator network through presentations and at national and international conferences. Both positive and negative data will be published in order to prevent unnecessary duplication of animal work, in accordance with the NC3R's ARRIVE guidelines. We will use our data in future grant applications for further scientific work and to obtain funding for clinical trials. We will share our computational models (we are already doing this nationally within our collaborator network) in order to ensure wider uptake of techniques and to increase the impact of the work. Through our clinical collaborators and a national advocacy program around the UK supply of radionuclides, we will work with cancer charities and patient groups within individual cancer types.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this project some normal healthy mice will be used (for control pharmacokinetic (PK) studies, for studying target expression in normal tissues, or for inoculation with mouse tumour cells). The majority of mice will be immune suppressed so that they can be implanted with human cells in order to study targets in human cancers. Some animals will be genetically modified to develop pancreatic cancer and this is to study animals that have more clinically relevant disease (i.e. developed over months rather than weeks) and within a normal immune system. Some rats will be used used (e.g. in cases where the normal target expression is more similar to that in humans, where pharmacokinetics differ between rats and mice or where a larger animal is required due to imaging resolution or mass dose constraints of e.g. radiosensitisers). All animals used in this project will be adult to decrease variability due to growth.

Typically, what will be done to an animal used in your project?

Animals will receive tumour cells injected subcutaneously (typically once, but occasionally one on each flank), intravenous tail veil injections (typically one, but sometimes one per week for up to four weeks). Animals will be anaesthetised using inhalation anaesthesia for minimally-invasive imaging (typically 30 min - 1 h duration) and this could typically happen once (sometimes twice) a day, but no more than 7 times in one week. Most animal imaging experiments are finished in 3 to 7 days when the animals are humanely culled and their tissues collected for analysis. One protocol allows for surgical implantation of tumour cells in the pancreas and this will be done under anaesthesia with analgesia given. More commonly this procedure will be carried out using a non-surgical image-guided injection of

cells into the pancreas. Some animals will also receive therapy by mouth or by injection (e.g. one dose or once a week for three weeks) and will be monitored for any side effects and also by in vivo imaging for disease regression/progression. Some imaging protocols require removal of food (typically for four hours) before administration of the contrast agent.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals should experience no more than transient discomfort due to injections or imaging. Tumour growth in efficacy studies may cause some discomfort or weight loss, but animals will undergo daily welfare checks for clinical signs and will be humanely culled to prevent any effects beyond moderate. The discomfort experienced with minimally-invasive imaging is due to repeated inhalation anaesthesia. This will be kept as light as possible to ensure fast recovery and animals will only be re-anaesthetised after complete recovery from the previous anaesthetic period. Animals experiencing tumour implantation via minor surgery (5%) will be given sufficient and suitable sufficient and suitable analgesia to temper any unnecessary pain during wound healing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

35% of mice and rats will be mild/low to moderate severity (e.g. subcutaneous tumour inoculation, one i.v. injection followed by culling).

65 % of mice and rats will be of moderate severity (e.g. in addition to tumour induction, undergoing more than one imaging event (cumulative severity increases from mild to moderate) and sometimes also surgery and/or therapy).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The work we are doing is to develop radiotracers and molecular radiotherapy agents that can be used to locate tumours in the body and treat cancer. These are administered intravenously to humans and are distributed round the body via the bloodstream. Tumour visualisation is highly dependent on how efficiently the tracer is retained in the tumour

while being cleared from the rest of the body. We need to use in vivo methods to determine the whole body biodistribution of new radiotracers, molecular radiotherapies, targeting agents and cell therapies to demonstrate visualisation of tumours or migration of therapeutic constructs through the bloodstream to tumour.

Which non-animal alternatives did you consider for use in this project?

In vitro methods such as cells or spheroids.

Why were they not suitable?

The studies being carried out in animals as part of this project are done only when selection of imaging agents and therapeutics has been performed using non-animal alternatives (for example, in cellular/spheroid models). These include the likely effect of the drugs or imaging agents on the target cells. However, the activity/uptake in cells cannot predict the behaviour of the drugs or imaging agents in a living body where they will interact with their target as well as undergo metabolism and excretion, therefore whole animals must be used.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our current and projected experimental program, we have estimated that around 2000 animals will be used in subcutaneous tumour radionuclide therapy studies over the next 5 years, researching around 10 new biomolecule-based constructs (approximately 2 per year) including control groups and comparison with standard biomolecules or treatments. These studies often involve detailed biodistribution pharmacokinetics in both males and females for dosimetry purposes in addition to imaging. We also expect to work on a minimum of three different T-cell based therapies during that time (500 animals over 5 years). A further 500 animals will be used in imaging-only studies (radiotracer development and response to therapy) in subcutaneous tumour models. In the case of tumours that develop deep within the body (orthotopic pancreatic mouse models, either implanted or transgenic), we will use 1000 over the lifetime of the project. Animal numbers in experimental groups are estimates based on the size of the effect that we expect to see between our various test conditions, standard agents and controls (using our own previous data, pilot data and/or that seen in the literature) and individual variability within groups, and will be different for each experiment. For example, receptor targeting studies in normal animals (where no therapeutic effect is expected) are usually carried out against a non-targeting (i.e. large difference in uptake in target organ between test and control) or

standard targeting molecule (i.e. smaller difference/equivalence or improved pharmacokinetics, reduction in off-target organ uptake).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Minimally-invasive imaging in live animals allows collection of data at multiple timepoints in the study, thus decreasing the numbers used compared to studies where groups of animals must be killed at multiple timepoints in order to collect data. Imaging studies can also allow each animal to act as its own control (similar to patient imaging studies) and allows paired comparisons. This increases the statistical power of experiments and decreases the number of animals needed, compared with terminal studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Better measurement of disease progression using imaging leads to a decrease in variability within groups (and therefore smaller groups). We will use a computational mouse atlas for image analysis which provides extremely accurate and fast estimation of tumours deep within the body.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For most of our imaging approaches, we will be using well-established cancer models. Nevertheless, tumour growth and development remains a key area of research to set up new predictive biomarkers, including imaging. This PPL particularly aims to target the early-mid stage of tumour development, and the combination of imaging and clinical assessment will support the implementation of comprehensive welfare approaches and early decisions on humane endpoints. In the case where tumours are developing deep within the body, screening using minimally-invasive imaging will allow us to detect tumours sooner and thus place animals into studies when their disease is less advanced, leading to a reduction in suffering. Imaging will be carried out under Inhaled anaesthesia wherever possible to minimise metabolic effects and achieve faster recovery times. Analgesia will be used for all surgical models.

Why can't you use animals that are less sentient?

The tracers and therapies we are developing and evaluating need to be evaluated in mammals before going into clinical trials. These animals should be metabolising normally during the study period in order that the pharmacokinetics of the agents are not altered.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In the case where tumours are developing deep within the body, screening using non-invasive imaging will allow us to detect tumours sooner and thus place animals into studies when their disease is less advanced, leading to a reduction in suffering. Imaging will be carried out under Inhaled anaesthesia wherever possible to minimise metabolic effects and achieve faster recovery times. Where possible, we will use implantation of tumours in the pancreas via ultrasound-guidance rather than open surgery, positioning the implantation in such a way so as to reduce constraint on the head of the pancreas . All surgeries/implantations will use appropriate aseptic technique and analgesia. Tumour-bearing and post-operative animals are monitored daily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Refining procedures for the administration of substances, Report of the BVAAWF/ FRAME/RSPCA/UFAW Joint Working Group on Refinement, D.B.Morton et al; 2001.

Guidelines for the welfare and use of animals in cancer research. Workman P et al, Br J Cancer. 2010 May 25;102(11):1555-77.

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Diehl et al, J. Appl. Toxicol. (2001) 21, 15–23.

LASA 2010 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. A report by the LASA Education, Training and Ethics section. (M. Jennings and M. Berdoy eds.). www.lasa.co.uk/publications.html

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have an effective 3R's network through the NC3R's (who fund some of our work) and attend our establishment 3R's events as well as those within our network. Staff attending conferences (such as the IAT Annual conference) will bring back best practice in terms of the 3R's and new techniques and we will keep abreast of the literature to ensure that we use the best and most reproducible methods.

46. Minimising tissue damage in electrosurgery

Project duration

2 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

polypectomy, electrosurgery, colorectal cancer, polyp lifting solution, Type II medical device

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to provide in vivo proof-of-concept that using a novel material ‘X’ leads to an improvement in the electrosurgical procedure compared to currently used procedure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Colorectal Cancer (CRC) is the third most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths in the world, and its burden is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030. In the UK it constitutes 11.5 % of the cancers and 10 % of cancer deaths are due to CRC. Most colorectal cancers start as a polyp and slowly grow into carcinoma over a period of few

years. The polyps larger than 2cm have highest risk; they are often sessile or flat requiring removal before development into CRC.

Polypectomy is the electrosurgery-based procedure used to remove polyps. It involves lifting of the polyp by injecting a solution underneath it (submucosal-injection (SMI)) so that it can be removed from the colon wall using an endoscope and electric cutting device without causing perforation of the colon. It is estimated that in the England alone around 600,000 polypectomies are performed every year. Perforation (1 -4 %), bleeding (0.5 -7 %) and partial polyp removal are major risks in polypectomy . The risk of post-polypectomy bleeding varies with polyp size, it is 0.4 %for small polyps and 5.3% for the larger ones. The odds ratio (OR) for the delayed bleeding was found to be 2.49 and 5.6 for the perforation for polypectomies involving large cecal polyps. The perforation risk for the large sessile polyps in the cecal region has OR of 12.19 and bleeding risk OR is 13.5. This high OR represents the cases where bleeding needs blood transfusion. It is estimated that in the UK heavy bleeding may occur in 60,000 cases (10 %) and perforation in around 6,000 cases (1 %) (https://www.hey.nhs.uk/patient-leaflet/advanced-polypectomy-for-large-polyps/), with global levels estimated as at least 20-times higher these rates.

Current practice in NHS, EU and many parts of the world involves inefficient practice using a mixture of Hypermellose eye drops and a dye injection (methylene blue or indigo carmine injection) solution. These SMIs are rapidly absorbed and require multiple injections to maintain polyp lift and offer minimal protection from thermal damage to the surrounding remaining tissue.

A research group at our Institution has developed a novel formulation ‘X’ which is a Type II medical device. 'X' has been shown in an ex vivo porcine stomach model to provide ~5x more polyp-lift than currently used SMIs, with a similar ~5x reduction in heating rate and thermal damage during electrosurgery.

Whilst these results in an ex vivo model are promising, they do not take account of tissue repair and the effects of disruption of the vascular network and at the site of surgery, and the next step is to obtain proof of concept in vivo, where repair can be more accurately modelled. The least invasive way of doing this so as to minimise distress to the animal is to use a skin model and this application seeks to test the formulation in a rat skin model.

What outputs do you think you will see at the end of this project?

The use of ‘X’ which forms the basis of this licence has the potential to overcome the current challenges with polypectomy surgical procedures, and the in vivo proof-of-concept studies carried out under this licence will validate the potential of this approach and allow further progress towards the clinic through late-stage preclinical and clinical trials. The output of the project will strengthen patent claims, and data which is not commercially sensitive will be used to generate research publications.

Who or what will benefit from these outputs, and how?

Ultimately the hope is that the ‘X’ solution being investigated here will translate to the clinic and benefit patients undergoing polypectomy, as the potential risks of undergoing the procedure will be reduced.

This will not be until beyond the timeframe of this project, with perhaps another four years of preclinical and clinical development required to realise this aim. The objective is to launch the product by 2025.

How will you look to maximise the outputs of this work?

The ‘X’ inventors will make the decision on the form of dissemination of any positive or negative information, which would comply with the ARRIVE guidelines.

Where we have any information that we can use that is non-confidential, then we will use this on our website and associated material to promote the work as a potential service going forward via social media, public audience events, and engaging with patient networks.

Species and numbers of animals expected to be used

- Rats: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rats are one of the most frequently used mammalian species for skin wounding healing studies and are selected here as opposed to the other common rodent model (mouse) as their larger size allows for more numerous and larger wounds to be studied. Adult rats will be used due to their larger size.

Typically, what will be done to an animal used in your project?

The in vivo model used in these studies is a cutaneous raised wound model. This is the least invasive way of evaluating the surgical technique in vivo and will minimise distress to the animal. Hair will be removed from areas on the back of the animal, & a small volume of solution injected sub-cutaneously (‘X’ at one site and a control solution at another site). The raised skin area (no more than 10 x 10mm) will then be excised using the thermo-electric cutting device & the wound covered with a loose dressing and allowed to heal for 2 months following the procedure, to give time for wound healing and scar remodelling to complete. The animal will be housed singularly until wounds have healed sufficiently to minimise risk of other animals interfering with the healing process, and then group housed The animal will then be euthanised and the wound area excised and examined histologically for extent of repair and any signs of tissue damage.

What are the expected impacts and/or adverse effects for the animals during your project?

Apart from potential discomfort due to inflammation at the site of surgery, which could potentially be moderate severity, and mild discomfort through repeated handling during the follow-up period, the rats will be able to live according to the 5 freedoms within the restrictions of being a laboratory animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that up to 75% of the animals will experience Moderate severity (the other 25% Mild). This is because 75% of the animals will be subject to surgical procedure where a small disc (10mm in diameter) is excised from the skin and then the healing process will be observed for 2 months, and therefore the animals will experience moderate discomfort as a result of the surgery, although this will be alleviated by the administration of post-surgical analgesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There are a few key reasons why it is necessary to use animals in order to evaluate the efficacy of the 'X' material under investigation here.

• Whilst results in an ex vivo model are promising, they do not take account of tissue repair and the effects of disruption of the vascular network and at the site of surgery, and the next step is to obtain proof of concept in vivo, where repair can be more accurately modelled.

• In addition, ex vivo assays do not take account of how the material behaves in a complex system,

i.e. are there interactions with surrounding tissue, circulating immune system cells and systemic toxicity away from the surgical site due to dissemination and degradation of the lift material.

•	Ethically it does not warrant proceeding directly from formulating a biomaterial in an ex vivo laboratory situation to evaluation in clinical trials in human patients.

Which non-animal alternatives did you consider for use in this project?

For efficacy, none, for the reasons given above

Why were they not suitable?

See above

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on carrying out a pilot study of 3 animals, followed by one extensive study (4 groups x 5 animals), with potentially a repeat of the study if borderline advantage (a trend of better efficacy but lacking statistical significance) was seen with the ‘X’ solution in the first experiment, and where a slight modification in the methodology, e.g. volume of ‘X’ administered, time of administration before surgery, would most likely improve the efficacy significance.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Reference to the literature and discussion with the biostatistician on the Institution’s AWERB and the inventors of ‘X’.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The use of pilot studies as mentioned elsewhere.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats are one of the most frequently used mammalian species for skin wounding healing studies and are selected here as opposed to the other common rodent model (mouse) as their larger size allows for more numerous and larger wounds to be studied.

We will always use aseptic technique and will commit to the LASA Guidelines as set out in 'Guiding Principles for Preparing for and Undertaking Aseptic Surgery' (2nd edition, April 2017).

Analgesics and anaesthesia will be used where required, and we will take advice relating to care and welfare via the AWERB and other appropriate sources.

Why can’t you use animals that are less sentient?

It is important to use a mammalian model, since this closer models for wound healing seen in the clinical situation, which would not be the case if a different Class is used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Analgesics and anaesthesia will be used where required, and we will take advice relating to care and welfare via the AWERB and other appropriate sources.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will commit to the LASA Guidelines as set out in 'Guiding Principles for Preparing for and Undertaking Aseptic Surgery' (2nd edition, April 2017), as well as referring to PREPARE and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through scanning most recent literature, referring to the NC3Rs and Norecopa websites and through information passed on by the local NIO.

47. Role for xxx in gene regulation

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

XXX, cancer, cell, gene expression

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the function of a protein called XXX, which is located in the nucleus of mammalian cells. When XXX is not produced correctly cells become abnormal and this can lead to cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When cells do not function correctly many different changes can occur that lead to a range of different diseases. Without XXX, or with the wrong type of XXX, cells can change and become cancerous. In fact XXX has been linked with many different types of cancer and other diseases, making it very important for us to understand how it works and what is going wrong when it is not working properly.

What outputs do you think you will see at the end of this project?

A significant amount of new information will be generated during this project improving our understanding of the role of XXX in controlling gene expression and DNA replication in normal cells during development. This will allow us to better understand what is going wrong in diseases with which XXX has been linked.

We expect a number of publications will arise from this work that we will submit to respected journals.

We will also generate information on gene expression and protein modifications that will be archived on publicly accessible databases.

Who or what will benefit from these outputs, and how?

These outputs will benefit other academic and clinical researchers:

Those working on control of gene expression and its contribution to disease. Those working on understanding how DNA is replicated.

Those working on understanding how the 'inactive X-chromosome' in female cells is kept inactive (male cells have 1 X-chromosome, whereas females cells have 2 X-chromosomes. One of these is shut down to stop the cell making too many gene products).

In the long term this work might also benefit the wider public, if information is found that could help to produce new medicines or ways to identify diseases at an early stage.

How will you look to maximise the outputs of this work?

We will continue to work with other researchers (collaborators) who help us to understand the full value of our work.

We will find new collaborators who have additional knowledge who can help us develop the research in new directions.

We will present the research at seminars and conferences at the earliest opportunity.

We will use the findings to help us prepare new applications for funding from charities and research councils.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cells in a growing embryo have the ability to become all of the different cell types in the adult body. As they become more specialized they gradually switch off genes that they no longer need until they are left making only the products that they do need. For example, a skin cell produces a range of products that are very different from those in a brain cell. If these fixed gene expression patterns are disturbed, the cell can begin to lose its identity and start to behave more like a cancer cell.

Our previous work has shown that XXX plays a role in controlling signals in the cell that help to control the expression patterns of some of these genes. In this project we will use genetically modified mice that do not produce any XXX. These mice also contain a second version of XXX that we have control over when it is made, by giving the mice an antibiotic in their drinking water. Addition of antibiotic leads to production of new XXX, while removal of the antibiotic causes XXX to be shut down again.

Alternatively, new XXX can be produced in cells that have been isolated from these mice and grown in culture, by adding the antibiotic to the culture media. This means that we can investigate what happens when new XXX is made in cells that have never previously made XXX. These mice (and cells isolated from them) are therefore extremely useful for helping us to understand what XXX is needed for.

We previously showed that a complete loss of XXX causes cancer (lymphoma) through overproduction of white blood cells in older female mice. However, in young mice the only changes reported to date are very mild changes in behaviour. For these reasons we will restrict our work to using mice up to an age of 12 months. Therefore, no signs of disease are expected. Much of the work will be on cells isolated from embryos. We can grow these cells in culture and apply a wide range of laboratory techniques to support our investigation. This will allow us to look for the earliest changes that contribute to diseases with which XXX has been linked, including cancer.

Typically, what will be done to an animal used in your project?

Use of timed matings will allow us to isolate embryos at defined timepoints, most usually at embryonic day 13. These embryos will be used for producing primary embryonic fibroblast cells that can be grown in culture. Addition of doxycycline to the drinking water (of pregnant females or post-natal mice) or culture media will allow us to produce new XXX in isolated tissues and cells derived from embryos and post-natal mice, whereas injection of substances such as ethynyl deoxyuridine (EdU) will allow us to monitor DNA replication in isolated tissues and cells. Liveborn mice will also be used for isolation of tissues at later stages (new born - adult). Some mice will be kept until early-mid adulthood (3-6 months). None will be kept beyond 12 months when the adult females are more likely to show symptoms of lymphoma.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect any adverse effects during this project, but if any are observed we will notify the NACWO and NVS immediately and appropriate action will be taken.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild (100%)

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The majority of the work will be done using primary embryonic fibroblast cells from genetically modified embryos (day 12-14) after timed mating. This means that animals are essential because these cells cannot be made in any other way.

Which non-animal alternatives did you consider for use in this project?

Immortalized mammalian cell lines that are able to be maintained forever under the right growth conditions, eg. mouse 3T3, and primary embryonic fibroblasts that we have produced previously, that have been cultured over a long period of time.

Why were they not suitable?

Immortalized cells are always abnormal and very often show characteristics of cancer cells. Our own studies have shown that cells obtained directly from embryos begin to lose their 'normal' characteristics within a few weeks of growing in culture. This means that they very quickly become unsuitable for many questions we want to ask. However, the same immortalized and long-term cultured lines provide an excellent source of material to study abnormal cells. We will use these unlimited sources of material as much as possible for testing new experimental methods before working with precious primary cells, to minimize animal usage.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For the range of experiments proposed in our funded grant application (valid 2021-2024) we require a minimum of 30 female primary embryonic fibroblast cell lines of each genotype (XXX modified and wild-type). Cell lines will also be made from male embryos at the same time to prevent any wastage. All lines will be preserved and frozen for use throughout the project, and made available to other researchers. The numbers have been determined based on known average litter size from interbreeding experiments, and for experiments requiring isolated tissues. They have been calculated by estimating those needed for:

Timed matings, with optional substance administration - 30 wild-type and 30 XXX modified (60, adult) Embryos needed for experiments - 60 wild-type and 60 XXX modified (120 embryos) Isolation of tissues, with optional substance administration - 60 wild-type and 60 XXX modified (120, various age)

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We consulted the Experimental Design Assistant (https://eda.nc3rs.org.uk), MRC and ARRIVE guidelines and constructed our plans in a way that uses the minimum number of animals to achieve statistically useful data. Experimental design regarding the use of cells and tissues was calculated using GPower (Mayr et al 2007), accounting for sample sizes (number of embryos required) and statistical analyses (which include both multivariate data and single parameter endpoints), where randomization is not appropriate for our experimental considerations, and measures put in place to avoid bias.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have made a genetically modified mouse that contains two 'transgenes' (additional genes inserted into the mouse genome). These two transgenes have been put into mice that do not produce any of their own XXX.

One of the transgenes contains a second XXX gene, and we have control over when it is turned on and off. The other transgene contains an 'activator' that is needed to control the XXX transgene. In the presence of an antibiotic in the drinking water (or cell culture growth media) the activator is able to turn on XXX gene expression. When the antibiotic is removed expression of the XXX transgene is stopped.

This means that we have extensive control over expression of XXX, from zero to high level, which can be turned on and off by addition/removal of an antibiotic. Breeding animals that contain these three components together allows us to produce useful

offspring at high level, with little or no wastage. We will endeavour to share tissues and cells from these animals with other researchers to further reduce usage.

Previous experiments with this combined genetic lineage have proven incredibly useful, resulting in high profile publications.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice that contain a controllable XXX transgene on a XXX deficient background. Extensive experience shows that these mice breed well and show no evidence of harm, suffering, distress or discomfort until late in adulthood.

Why can’t you use animals that are less sentient?

We are asking questions about how cells fine tune regulation of gene expression during mammalian development and disease, so it is not possible to use non-mammalian species that are too diverged in regulatory mechanism. Focussing the majority of our investigations on primary embryonic fibroblasts derived from embryos means that we are using animals (mice) at a very immature life stage. Killing of the mother prior to embryo retrieval means that harm, distress and suffering will be kept to a minimum.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible environmental enrichment will be adopted, following accepted procedure. Handling of the mice will be aimed at encouraging minimal stress and discomfort, using tube handling refinement technique where possible. Animals will be examined daily and any signs of ill health dealt with promptly through discussion with the NACWO, and NVS where needed. Careful monitoring of timed matings will facilitate accurate estimation of embryonic stage which is critical for our experiments. This will keep number of timed matings required to a minimum. When plug check is inconclusive females will be weighed daily to look for evidence of rapid embryo growth, which provides good evidence of mid-gestational development.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

National Centre for the Replacement, Refinement and Reduction of Animals in Research website (nc3rs.org.uk). eg. https://nc3rs.org.uk/3rs-advice-project-licence-applicants-refinement

PREPARE guidelines: https://norecopa.no/PREPARE Laboratory Animals Science Association (lasa.co.uk)

re routes of administration https://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf ARRIVE guidelines (arriveguidelines.org)

Eg. The design of animal experiments (Festing, Overend, Cortina Borja, Berdoy) SAGE publishing

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay up to date with guidance from the Home Office, including updates to the following resources:

National Centre for the Replacement, Refinement and Reduction of Animals in Research website (nc3rs.org.uk). eg. https://nc3rs.org.uk/3rs-advice-project-licence-applicants-refinement

PREPARE guidelines: https://norecopa.no/PREPARE

Guidance of the Operation of the Animals (Scientific Procedures) Act 1986

Regular interaction and feedback from the NIO, NACWO, NVS and, where required, the Home Office inspector.

Guidance will be followed at the earliest stage possible.

48. Brain development and neuronal network maturation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

brain, development, neurogenesis, synapses

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to reveal how genes and the environment guide and modulate the formation of the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The brain is the most complex of the body's organs and its role in generating behaviours has fascinated generations of researchers. The huge complexity of the adult brain can be resolved by studying the process of brain development, when its organisation is still relatively simple. Because perturbations of the normal process of brain development result in alterations of the mature brain's structure and function, a detailed understanding of brain

development is also required to tackle the causes of neurodevelopmental disorders, such as autism, schizophrenia and epilepsy.

What outputs do you think you will see at the end of this project?

This project will lead to novel discoveries on the fundamental principles of brain development and maturation. The project will generate new data that will be published in peer-reviewed scientific journals, for the benefits of the scientific community.

Who or what will benefit from these outputs, and how?

The project will generate peer reviewed publications during the course of its implementation and in the years that follow its completion. The scientific community will benefit from the data generated by this project and use them to build new theories and experimental hypotheses, therefore moving forward the frontiers of biomedical research.

How will you look to maximise the outputs of this work?

I have in excess of 20 years of biomedical research using rodents and a track record of successful peer reviewed publications, international conference presentations, book chapters and international collaborations and exchanges with some of the major research institutes in the world working on neurodevelopment, including in Japan and the US. These activities will continue to ensure that the output of this project reaches its intended beneficiaries. At local institute level, there is high potential for synergy, with several lines of research complementary and relevant to the one presented here. Over the course of this project, we will facilitate transfer of knowledge within our local community for mutual benefit and also to avoid potential duplication of procedures. For instance, data generated on the type of genes expressed by different cells of the developing brain can be shared and used by others, often without the need to replicate the steps that involve animal handling.

Because the project is focused on the description of newly identified cell types, it is unlikely that there will be unsuccessful outcomes - any new data will be useful data and will be published, compatibly with scientific rigour in data presentation.

Species and numbers of animals expected to be used

- Mice: 5100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The primary goal of this project is to study the mechanisms that explain the organisation of the mammalian brain, hence a mammalian system is required. Genetically altered mice offer the advantage of studying well defined cells and tissues, helping to disentangle the complexity of the brain. No other mammalian system offers the same palette of genetic tools. The project is largely focused on early life stages: foetal and pre-weaning. In some cases, when information on the synaptic organisation of neurons is required, then adult animals will be used. In such cases, animals are culled within 6 weeks from the start of regulated procedures.

Typically, what will be done to an animal used in your project?

Animals carrying genetically modified alleles may be bred and culled for tissue collection. Some pregnant animals are used to perform manipulations to the embryos in utero.

Some pregnant animals and their offspring may be exposed to environmental challenges, such as non- pathogenic surrogate of viral infection or vitamin depleted diet.

Some animals are used to inject intracranially fluorescent tracers that reveal neuronal connections.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect clinical manifestations of pain, weight loss, tumours in this project. However, transient pain and weight loss may occur. Phenotypes are expected to be subtle and only detected at the microscopic level. As a result of developmental changes in teh brain cognitive and social interaction behaviours may change too, however, in this project, animals are not generated for the purpose of studying animal behaviours, but rather cellular and molecular alterations and will therefore be culled before weaning or at 6 weeks of age at the latest.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

A mild severity is expected for breeding protocols. All other protocols are expected to be moderate. Exposure to moderate procedures affects approximately 50% of the animals used in this project.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brain is composed of hundreds of different classes of classes of cells eac each class with specific functional roles. The complex behaviours that define the ability of an animal to survive in its environment and to establish social interactions with other animals in the same group emerge from the coordinated action of the many cell classes that compose the brain.

Cell classes in the brain are defined by expression of selected genes during brain development in gestation that control their differentiation into mature neurons (the main cell type responsible for information processing in the brain). The spatial organisation of different cell classes in the brain, which results in the anatomical organisation of this organ, is of fundamental importance to enable but also to constraint brain function.

Such degree of complexity and dependence on the full 3D anatomical organisation of the brain, impose that research is performed on a living, intact organism.

We relied on the support from the Fund for the Replacement of Animals in Medicals Experiments (FRAME) to identify alternative strategies to the use of protected animals when planning our experiments, but found that no alternative in silico or tissue culture system would be available to achieve our research targets.

We feel that the use of a genetically altered mouse model would allow us to study the interactions between genes and the environment that guide brain development and maturation. the genetically altered mice that we will use allow to selectively target cells of interest on the background of an otherwise normal tissue. This approach will produce data that faithfully describes biological complexity and has the potential to truly advance knowledge in the field of mammalian brain development.

Which non-animal alternatives did you consider for use in this project?

We considered the use of in silico models and of tissue culture organoids. We have also considered the use of ex-vivo live tissue analysis.

Why were they not suitable?

In silico and cultured tissue models cannot reproduce the spatial and temporal complexity of brain development. While organoids (when stem cells self assemble in a simpler form of an embryonic tissue in a culture dish) hold promise, they are not suitable to study the establishment of neuronal networks, because development only recapitulates the early events of brain tissue specification. We plan to make use of in vitro systems when possible, for eample, we will be able to monitor the movement of cells in brain tissue for short periods of time, using ex-vivo tissue culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have estimated the number of animals to be used based in this project based on actual animal usage from the previous 2 project licences that I have held. As the general area of research, level of research funding and number of personal licence holders working under this project licence are expected to be in line with the previous years, the number of animals to be used can be estimated with a high degree of confidence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This project relies on the use of several GA mouse lines, typically, mutant alleles, cre-recombinase expressing lines and conditional fluorescent reporter gene lines. COnditional reporter lines are activated by crossing with a suitable Cre-expressing GA line. We will maintain minimal stocks of stud males with heterozygote cre and maintain the conditional reporter lines as homozygote. We will not keep these animals in breeding unless ready to conduct an experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The largest fraction of animals in this project is to be used to study small groups of cells in the brain. While targeting such small brain areas used to carry a high degree of variability and hence require many replicates, we base all our work on the use of genetically modified mice that express the cre- recombinase under cell type specific promoters and this gives a very high level of anatomical precision to our manipulations that invariably cut the number of animals needed to obtain statistically robust data. By choosing to work with cre mouse lines, we can drastically increase the specificity of our brain injections to target specific neurons of interest, as only cre-expressing neurons would respond to the treatment/manipulation.

For embryonic manipulations, in previous work we have practiced and refined techniques over the past 10 years and established protocols on the CD1 mouse strain, known to carry a higher number of embryos than C57Bl6, therefore maximising the number of experimental samples (embryonic brains) per dam. This strategy reduces considerably the number of dams and capitalises on work done under previous licences to define optimal sample concentrations and target areas for the neurons of interest.

We will perform our histological analysis on serial sections so that one biological sample will serve for multiple experiments. All spare tissue samples are stored long term in a special solution at freezing temperatures so that tissue material can be analysed months and years after the animal was culled, eliminating the need to each time generate new experimental animals.

For studies of neuronal cell migration, we have developed an ex vivo brain slice system that gives us a time window of 1-3 days to follow the migratory behaviour of fluorescently labelled neurons in vitro. This approach allows us to cut the number of mice that would otherwise have to be culled at short time intervals to obtain similar time-lapse information that we can now have with just one tissue sample (i.e. one mouse).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model of choice is the mouse. In particular we will use genetically altered mouse lines, which provide much increased specificity of genetic manipulations, reducing the need for a high number of replicates (animals) used in each experiment.

This represents already a step towards refinement: in the past, studies that aimed at correlating anatomical brain structures with animal behaviour relied upon open scull surgery on larger mammals, such as ferrets, hamsters and cats. In these procedures large areas of the brain were damaged or removed by mechanical means including aspiration. While a small mammal, such as the mouse is ideal for our research, the use of lower organisms such as Drosophila (insect) and zebrafish (fish) does not offer the same complexity of brain circuitry for the ultimate goal of understanding human brain function.

Why can't you use animals that are less sentient?

The nature of our research is naturally focused on early life stages: embryonic, newborn and juvenile, when the nervous system is still being formed and refined and therefore the impact on an animal welfare much reduced compared to performing the same procedures on adult animals. For instance, we have developed and regularly use a protocol to perform stereotaxic brain injections at 2 weeks of age, typically resulting in the end point (animal culled) 3-6 weeks later. This represents a significative temporal compression of the procedure compared to standard methods.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have modified the standard procedure for stereotaxic brain injections to use extra-fine glass needles instead of Hamilton syringes which has the benefit of requiring only a pinhole in the skull bone and generates negligible mechanical brain damage due to the fine diameter of approx. 100 micron.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow guidance available on the "resources" page of the National Centre for the Replacement, Refinement and Reduction of Animals in Research at https://www.nc3rs.org.uk/3rs-resources

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will abide by the institutional reviewing procedures already in place such as periodic reviews of the project licence, regular discussions with the NVS and by regularly consulting the web page of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://www.nc3rs.org.uk).

49. Airway fibrosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

lung fibrosis, IPF, Pharmacology, rodents

Animal types	Life stages
Mice	adult, aged
Rats	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Understanding the biology of airway fibrosis in experimental animal systems and using that knowledge to identify and develop novel treatments for human fibrotic airway diseases

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Fibrosis is the term used to describe the thickening, stiffening and scarring of parts of the body that can happen in disease. Despite decades of research, there is still a need to discover and develop new medicines for fibrosis. One serious disease of this type is called

idiopathic pulmonary fibrosis (IPF), and patients with this develop progressive lung fibrosis with a higher mortality than many cancers, and only two therapies are currently available (Ovef® (nintedanib) and Esbriet® (pirfenidone)), both of which only slow disease progression by approximately 50%. These medicines do not halt disease progression, but do provide a clear mortality benefit and yet have significant side effect profiles which impact patients' quality of life. Sadly, this position has remained unchanged since the writing of the preceding licence 5 years ago.

What outputs do you think you will see at the end of this project?

Our ultimate goal is to see new therapeutic agents progress into the clinic and be used to treat patients with fibrotic disease. The decision to move into clinical phase testing requires a great deal of information from multiple sources including human tissues, cells, safety and pharmacokinetic data as well as key data from suitable animal models. The outputs from this licence specifically could include pharmacodynamic information on the ability of the novel treatments to modulate lung fibrosis after bleomycin or TGFbeta administration, as measured using biochemical or histological/histochemical methods. Other readouts could include assessments of fibrosis relevant gene expression (such as collagen genes) or other pathways including senescence, inflammation or oxidative stress. We hope that at the expiry of this licence we will have progressed at least one novel treatments for human airway fibrosis into early clinical testing.

Who or what will benefit from these outputs, and how?

The new knowledge we will create, for example on the impact of novel therapeutics on the development or regression of lung fibrosis will contribute to the overall package of information needed for go/no-go decision making ahead of clinical stage testing. This information will also be of great use to the wider scientific community and will be promptly shared whenever possible, for example as conference abstracts, and could enable other new medicines and treatments to be generated. We would expect to share information on model development or refinement with the scientific community rapidly and without limitation. We expect the new medicines we will discover and develop to have significant positive impacts on the lives of patients, their families and society as whole. These benefits may not be tangible until 10-15 years after initial discovery, so they are unlikely to be measurable during the lifetime of this licence.

How will you look to maximise the outputs of this work?

We will share the new knowledge whenever we can through publications, conference posters and presentations. We have networks of academic collaborators with whom we share our findings and benefit from the discussion and validation of our findings. We are aware that there is a need to share negative findings as well as positive ones, and we will engage with others in the scientific community to understand if there may be ways to share non-commercially sensitive information that might reduce animal usage.

Species and numbers of animals expected to be used

- Mice: 2000
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult and aged rats and mice are appropriate animals to study respiratory diseases as they have anatomically and physiologically relevant mammalian biology. Adult animals (>6 weeks old for mice, >8 weeks old for rats) are fully formed and can demonstrate representative tissue and immunological responses to inflammatory or other stimuli. Aged animals (>15 months for mice, >1 year for rats) may be used as we recognise that in some respiratory diseases (such as chronic obstructive pulmonary disease (COPD)) age itself may be a factor that limits the ability of the lung to repair or regenerate and we wish to understand mechanisms that could surmount this.

Typically, what will be done to an animal used in your project?

A typical study may involve 6 groups of 8 mice or rats, which might be identified using a subcutaneous microchip after arrival, randomisation into cages and acclimation. At least 5 days after delivery animals would have a fibrotic stimulus administered into the lungs in a small volume of liquid (under anaesthesia). Animals would be returned to their home cage once recovered from anaesthesia, closely observed to ensure they are not exhibiting any unexpected side effects, checked again at the end of the working day and then left overnight. From this point on animals would be checked and weighed daily to monitor weight loss that is expected due to the inflammation in the lungs. Treatment to modulate the fibrosis would be given, for example by a twice weekly injection or daily oral dosing, for 2-3 weeks, and at the end of the study the animals killed humanely and the lung tissues taken for analysis of fibrosis.

What are the expected impacts and/or adverse effects for the animals during your project?

Induction of fibrosis of the airways is likely to be uncomfortable and animals may exhibit clinical signs of discomfort or illness such as loss of appetite, reduced grooming and reduced social interaction.

Reduced food intake can lead to a body weight loss, and for this reason animals are weighed daily after induction of fibrosis. Animals with weight loss are expected to recover after a few days, as normal feeding resumes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice and rats:

We expect approximately 90% of animals to be classified as Moderate, the remaining 10% being Mild. This will depend on the specific studies but in many cases we will have negative (non-fibrotic) controls who will not develop fibrosis or experience weight loss, as well as positive control diseased animals (fibrotic, untreated) to enable an appropriate comparison. The remaining groups would be treated with new experimental materials (or their dosing vehicle as a control), which we would be expecting reduce the disease and thereby reduce suffering. Acute weight loss is observed in lung fibrosis models using bleomycin or TGFbeta, and the dose-response for this effect is very steep and often difficult to separate from the dose required to induce fibrosis. Weight loss is usually less than 20% at maximum (~15%) at 7-8 days post challenge, and animals will recover weight. Some animals will transiently reach the 20% limit and then recover, any animals that do not recover weigh after 24 hours are removed. The proportion of animals that reach the 20% limit varies between study, despite identical material, operators and mice source/age. In the last 3 bleomycin studies using C57/Bl6 mice the number of animals withdrawn due to weight loss was 13/32, 7/48 and 10/38 (ratio shown as a proportion of animals receiving bleomycin, not the full study size including non-fibrotic controls)

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Respiratory diseases arise through complex interactions of multiple biological, genetic and environmental factors. The structural cells of the lung tissue, the underlying vascular and nervous system components, and the immune cell populations all interact in ways which cannot yet be modelled with sufficient predictive power to replace animal studies, especially when novel mechanisms which are not fully understood are involved.

Which non-animal alternatives did you consider for use in this project?

Non animal systems are a key part of our research programmes, and animal studies are only considered when sufficient data are available to justify their use or in certain circumstances where the biology is novel and not characterised sufficiently to enable in

vitro emulation. Non-animal systems we use include cell culture models (including organoids) which can be generated from primary human tissue or immortalized cell lines. Studies in these model systems can enable early validation of hypotheses and testing of novel therapeutic approaches.

Why were they not suitable?

The in vitro systems we do use are not complete representations of the lung in health or disease. Cell culture systems, even when they have some structural homology (such as organoid culture) do not have the complex biology seen in living organisms where multiple types of cell and structure combine. Human tissue, when available, is not a complete surrogate as it lacks exposure of the lung cells to air (in submerged culture), blood circulation, innervation, immune cell trafficking and many other components.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates are based on anticipated throughput of studies and using numbers typically used in previous experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Study designs are reviewed as part of our internal statistical review system to ensure that studies are appropriately constructed to account for biases and are sized to enable robust conclusions to be drawn. Statistical techniques including power analysis are used to ensure that hypothesis testing studies are correctly sized. Studies are normally conducted for a single project to enable sufficient tissue for any project-specific assays, and to allow for a dose response design. When possible we will try to combine studies to reduce the number of control groups across the licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Much work is conducted in vitro on cells and human tissue prior to human work, which reduces animal usage for preliminary experiments. Breeding of rats and non-genetically modified mice is performed for us at a commercial supplier which enables the maximum efficiency of animal usage for these animals. Genetically modified mice are not bred under this licence but are supplied under continuous use authority. The breeding schemes for

these lines are closely monitored and for established lines with straightforward genetics (e.g. a single knockout or manipulation of a single locus) homozygous breeding is used as much as possible to reduce wastage significantly (as 100% animals have the correct genotype rather than 25% for heterozygous breeding). Where possible we will use pre-exisiting information on compounds to correctly power study designs. Due to the risk of animals being withdrawn due to weight loss, group sizes may be adjusted upwards to avoid repeat studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use belomycin and adenoviral delivered TGFbeta models of airway fibrosis in rodent species (mouse and rat). These will enable us to study the mechanisms involved fibrosis, especially in relation to human lung fibrosis, which is present in diseases such as idiopathic lung fibrosis (IPF). These methods of inducing experimental fibrosis can also cause airway inflammation which contributes to the fibrotic changes to the lung tissues and reflect what is seen human diseases, so it is likely that animals will experience respiratory changes and discomfort that cannot be avoided. However, as we are seeking to find and develop new medicines, some animals would be likely to have this suffering ameliorated by our novel treatments. We have selected airway models that are well tolerated and are expected to result in a moderate level of severity, which includes some clinical signs such as reduced food intake and lack of grooming. Where possible we will carry out studies using anaesthetised animals that can be immediately killed without recovery, to minimise suffering.

Why can’t you use animals that are less sentient?

We are seeking to identify and develop treatments for use in human lung disease and therefore although there may be some analogous systems for gas exchange in lower species such as flies, worms or fish there are large differences in anatomical structures that make extrapolation to human disease very difficult. In addition we would not expect therapeutic agents which have been developed to work against specific human receptors or mediators to have the necessary efficacy in these lower species as they are evolutionarily more distant from human compared to rodents. Rats and mice have an anatomically similar (but not identical) lung and respiratory structure to humans, and have a more similar immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals are closely monitored and are kept in modern purpose built facility with highly trained and motivated technical staff. We implement many systems to improve animal welfare, including non- aversive handling (i.e. not picking up by the tail), environmental enrichments such as tunnels, nesting materials and chew sticks, as well as cage floor level access to food for animals displaying reduced activity or mobility.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice, for example the use analgesics after surgical procedures will be employed to minimise suffering.

Publications such as the Handbook of Laboratory Animal Care and Welfare (Wolfenson and Lloyd), and the LASA best practice guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am in regular contact with other in vivo scientists and teams of animal welfare specialists within and outside my organisation.

50. Hypoxia, carbon dioxide and acid sensing in respiratory chemoreceptors.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Chemoreceptor, Carotid body, Hypoxia

Animal types	Life stages
Mice	neonate, juvenile, adult
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To advance our understanding of oxygen and carbon dioxide sensing in respiratory chemoreceptors. To identify other signalling pathways and drugs that can also modulate chemoreceptor activity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Control of blood oxygen and carbon dioxide levels is essential to sustain life. This requires sensors that can measure these variables and through respiratory control centres in the brain regulate breathing.

What outputs do you think you will see at the end of this project?

We expect significant advance in our understanding of the mechanisms of oxygen sensing in this tissue and hopefully some insight into how the activity of chemoreceptors may be controlled by other pathways.

Who or what will benefit from these outputs, and how?

The primary benefit will be the advancement of science in the carotid body field. This will not only be of interest to others working in this field but possibly also in the field of hypoxia sensing in pulmonary blood vessels. Hypoxic pulmonary vasoconstriction shows many similarities to hypoxia sensing in the carotid body. Together they play a key role in helping to maintain oxygen levels in the arterial blood.

Both have implications for the understanding of hypoxemia in a clinical environment (possibly including Covid 19) and in life at altitude.

Our studies may also point the way to the development of strategies for controlling chemoreceptor activity pharmacologically. This may be of benefit in hypertension, wherein increased chemoreceptor activity leads to increased activity in sympathetic nerves which may feedback to generate a further neurogenic component of hypertension. It may also be of benefit in anaesthesiology where both anaesthetics and opiate analgesics blunt chemoreceptor responses to hypoxia and depress breathing in general. This increases the risk of postoperative hypoxemia and may limit the effective use of opiate analgesics for pain management (for fear of depressing breathing further).

How will you look to maximise the outputs of this work?

We have ongoing collaborations with other groups on the biology of chronic oxygen sensing, acute oxygen sensing and the effects of anaesthetics and other drugs on chemoreceptor function. New knowledge will be disseminated through publication of primary research, review articles, and presentation at scientific meetings. Publication of negative findings will also be presented in the same manner where it helps answer specific scientific questions and where reliable conclusions can be drawn. It may be more difficult to publish where techniques have simply failed to work.

Species and numbers of animals expected to be used

- Mice: 1700
- Rats: 800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are chosen where we need to use gene modification techniques.

Where we do not need genetic modifications we will mostly use neonatal rats. Rats are preferred over mice as the organs we are interested in are larger and tissue from neonates is easier to dissociate into cell cultures. This means we can use fewer animals. Most of our research is conducted in vitro using cell cultures. The rat model is also better studied.

Typically, what will be done to an animal used in your project?

Removal of tissues under non recovery anaesthesia.

Administration of some agents used to induce genetic modifications. This is usually via an oral route over a period of 5 - 7 days.

Measurement of an animals breathing responses to changes in oxygen and carbon dioxide levels in the air by a non-invasive method. Over a period of 3 hours on up to 8 separate occasions.

Acclimatisation of animals to chronic hypoxia over a period of up to 2 weeks. To simulate ascent to altitude.

What are the expected impacts and/or adverse effects for the animals during your project?

Weight loss due to chronic hypoxia and/or administration of agents to induce genetic modifications. Over several days.

Increased breathing with possibility of brief dyspnoea (air hunger) during exposure to acute hypoxia (for 5-15 min).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Rats. Non-recovery anaesthesia.
- 100% Mice. Nonrecovery anaesthesia.
- 10% Mice. Mild. 60%
- Mice. Moderate. 30%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The process we are investigating involves a highly specialised function of one organ and probably only one cell type within that organ. We do not know of any cell line with the same capability.

Which non-animal alternatives did you consider for use in this project?

We do not know of any non-animal alternatives. In some cases, where we have identified a specific protein involved in chemoreception, we can study some aspects of that protein's function when it is expressed in a cell line. But as it is in the wrong cell type other components of the relevant signalling pathways may be absent. So the usefulness of such models is very limited.

Why were they not suitable?

Unable to replicate responses to hypoxia similar to that seen in native carotid body tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The vast majority of the research to be conducted involves experimentation done in vitro and so this the biggest user of animals overall. Our estimate of the number of animals used in in vitro experiments is based on the past experience of myself and others in performing these types of experiments their complexity and level of difficulty e.g.

Does it require one or more control groups or can we rely on a control measurement made in the same cell (e.g. are we comparing different genotypes).

Can the objective be achieved with a single stimulus or do we need a full dose response (e.g. defining oxygen sensitivity of mitochondrial function).

Productivity of the cell isolation process (much better in rats than mice).

Level of difficulty in applying the technique itself (success rates are much lower using electrophysiological techniques than when using imaging techniques).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The majority of research to be conducted in this project will involve removing tissue from a donor animal and breaking it up into its component cells. Experiments are then performed on these cells in vitro. With careful planning cells obtained in this manner can effectively be used for a number of different experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Given that the majority of work is conducted in vitro this is largely about productivity in cell isolation and the success rate of the in vitro techniques used to measure cellular events. We endeavour to optimise the techniques used for cell isolation to minimise the number of animals needed to generate each cell isolate (typically 2 animals). We also aim to maximise the technical success rate of each in vitro technique, this is largely achieved through experience and fine tuning of research protocols and methods. Finally, we can conduct more than one type of experiment on each cell isolate. So typically, we might plan to perform 3 different experiments, each with 2 technical replicates, using one single isolate thereby generating 3 pieces of experimental data for each animal used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used where we can utilise genetic modifications to help understand/elucidate signalling pathways involved in the processes of oxygen sensing.

Where we do not need genetic models we mostly use rats because their carotid bodies are larger than in mice and can be used to prepare cell cultures more readily (requiring fewer animals). Most of our experiments are conducted in vitro using such cell cultures.

The main form of in vivo experimentation is measurement of ventilation/breathing. This is conducted by placing the animal in a small chamber and recording pressure changes in

the chamber as the animal breaths in and out. This technique is called whole animal plethysmography. This is a non-invasive technique which causes minimal distress to the animals.

Some animals are acclimatised to chronic hypoxia in a large chamber fed with air with a lowered oxygen content using a hypoxicator. This is a device often used by athletes to improve their performance. Animals are housed in groups in standard cages which are then placed in this chamber.

Why can't you use animals that are less sentient?

The response of the carotid body to hypoxia is blunted in new-born animals and does not show normal sensitivity to hypoxia until animals are at least a week old. There is little information on chemoreceptor function in lower vertebrates, e.g amphibia and fish, so we do not know whether they would be suitable replacements or not. Any data obtained from such animals would also have to be validated in a mammalian model so there is little to be gained by substituting these animals in this type of research.

Most of our research is conducted in vitro using tissue obtained from terminally anesthetised animals.

The main in vivo experimentation is plethysmography. We cannot measure breathing responses to hypoxia under anaesthesia because many anaesthetics depress breathing and in particular inhibit responses to hypoxia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Regular monitoring of animal health for transgenic animals and animals subject to chronic hypoxia.

Continuous monitoring of animals undergoing measurement of breathing responses to brief hypoxic and carbon dioxide stimuli for any signs of respiratory distress or loss of coordination.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will refer to NC3R, LASA, RSPCA and ARRIVE guidelines. I will also monitor published research in this area for examples of improved techniques

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will regularly review/search the NC3R's website and news letter for relevant information. I attend a regular internal animal welfare meeting (Gold Standard meetings). I also perform regular literature searches relating to this field of research.

51. Assessment of bioaccumulation in fish

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Bioaccumulation, Regulatory, Safety, Chemical, Pharmaceutical

Animal types	Life stages
Rainbow Trout	juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of the project is to determine the bioaccumulation of industrial chemicals, agrochemicals, pharmaceuticals and biocides in fish such that the hazardous properties of these substances with respect to their ecotoxicological (effects on the environment) properties can be assessed. These properties are a fundamental requirement of the risk assessment process for such substances, and are there to protect humans, animals and plant life.

These studies are required by law by global regulatory authorities on behalf of governments to protect humans and animals from the potential harmful effects of these chemicals.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The need for these studies is driven by regulatory requirements to provide data on the potential bioaccumulation of test chemicals in fish with the study data being used to support the registration of the new chemical or preparation, as required by the prevailing notification/authorisation scheme in the countries where it is to be manufactured or imported. These tests are required by law around the World. These tests are required to keep the environment, animals and humans safe.

The studies are conducted in accordance with scientifically relevant and internationally approved test methods so that the data will be acceptable for registration or notification of the chemical or preparation in regulatory schemes such as REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) for chemical notifications, European Medicines Agency (EMA) for pharmaceuticals and European Food Safety Authority (EFSA) for agrochemicals.

What outputs do you think you will see at the end of this project?

The main benefit of this project is the development of data to support the risk assessment of chemicals such that any detrimental effects on the environment can be minimised. This will protect the environment, animals and humans.

This will allow regulatory authorities (organisations independent of governments who assess the safety of a variety of drugs and chemicals) to classify and label these substances, recommend safe handling procedures, and impose risk reduction measures if required such that the benefits provided by the substances can be safely achieved.

Who or what will benefit from these outputs, and how?

The main benefit of this project is the development of data to support the risk assessment of chemicals such that any polluting effects on the environment can be minimised.

This will benefit the environment, the public, wild animals and the people who produce these chemicals (as it will enable them to be registered and sold to the public or industry).

How will you look to maximise the outputs of this work?

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for regulatory purposes or to support regulatory purposes (e.g. to show that a certain chemical is safe for human exposure). Previously however, we have collaborated with customers and shared data we have produced in the form of Scientific publications that are in the public domain.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project uses freshwater fish (rainbow trout, common carp and bluegill sunfish) to assess the bioaccumulation of industrial chemicals, agrochemicals, pharmaceuticals and biocides that may end up in the environment in a living organism.

The fish chosen are based on the regulatory requirements of the test. Juvenile fish are chosen, and the age ranges of the fish are such that they are the youngest fish that have a fully functioning brain and nervous system that will allow evaluation of the specific types of data we need to fulfil the requirements of the test.

Typically, what will be done to an animal used in your project?

The individual studies undertaken involve exposure of groups of fish to varying concentrations of the chemical to assess the bioaccumulation of the chemical in the fish tissue. This can be done by adding the chemical into the water directly or adding it to the food the fish eat.

Following on from the bioaccumulation phase (where the chemical can accumulate in the tissues), a secondary phase where the fish have no exposure to the chemical is undertaken. This is conducted (where appropriate) in order to determine whether a reduction of the bioaccumulated chemical occurs in the fish tissues (this is called depuration).

Any fish that are exhibiting adverse effects which are in excess of the mild severity level will be humanely killed as soon as possible to avoid unnecessary suffering. In order to measure the bioaccumulation within the fish, fish will be humanely killed on a regular basis throughout the study to enable chemical concentrations within the fish tissue to be determined (specifically in the fat).

All fish that are killed during the study and any that remain at the end of the test will be humanely killed by a method approved by the government.

What are the expected impacts and/or adverse effects for the animals during your project?

The tests are designed to be performed at concentrations below those which are predicted to cause adverse effects on the fish and hence in the majority of exposed fish no adverse

effects will be seen. Experience suggests that in less than 5% of exposed fish will any mild adverse effects occur.

During these studies, the fish are watched closely after exposure to a chemical. We have a list of adverse clinical signs; our staff are experienced and well trained in what to look out for and noticing abnormal behaviour. If we see any adverse signs, we increase our observations until they pass, or if these signs become worse than mild to moderate, we will remove the fish and humanely kill it to prevent any further suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

As we are using concentrations that are predicted to be below those that would be expected to cause adverse clinical signs, we would only expect 5% of animals to display mild to moderate clinical signs.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Current regulations, e.g. REACH, require the use of fish to assess potential environmental effects of chemicals.

Non-animal alternatives (in test tubes or using tissues from dead animals for example) have not yet been sufficiently validated for acceptance by various regulatory authorities and hence cannot be used to replace animal testing in this context.

In many cases the protocols listed in this Project will be used later in the life cycle of the testing of a chemical and in many cases, tests in test tubes will have been conducted previously (often by the Sponsors) to examine their potential toxicity in cells, before we expose them to testing in animals.

Which non-animal alternatives did you consider for use in this project?

There are no other non-animal alternatives for the work being undertaken on this project.

Why were they not suitable?

There are no adequate models to replace the whole animal experimental model, as the complexity of fish and their internal physiology and development cannot be fully replicated in a test tube.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals, and the number of groups of animals to be used in a particular test are usually defined within the regulatory guideline used for that test. No more animals than the numbers outlined in these guidelines will be used. The use of the specified numbers of animals ensures that the data generated will be acceptable to regulatory authorities and hence will minimise the need for subsequent duplication or supplementary testing.

Whenever possible, common control groups will be used in order to minimise the numbers of groups used.

We also have a preliminary test that helps us set exposure concentrations and test conditions, that if is successful, means we don't have to perform any more testing on fish to satisfy the regulatory needs for a chemical.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Some of the studies run in this project will automatically use data from previous experiments to inform on exposure concentrations levels for future studies. Therefore it will not be necessary to run another study, using more animals, when we already have that information. Similarly, if we have that data from other sources, we don't need to run these preliminary range finding studies.

Where possible the results of computer predictions, physico-chemical testing and non-animal tests will be used to aid in the prediction of toxicity hence reducing the number of animals required to satisfy the regulatory requirement.

We will try and get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to get a blood sample, or if we need to get a fat sample, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The fish species selected (trout, carp, bluegill sunfish) are representative of wild species. The data generated is therefore designed to protect these representative species in the environment thereby minimising larger scale environmental effects of tested chemicals.

Our models involve exposing fish to test chemicals, by direct exposure in the test water or via their food and observing them for signs of toxicity.

Many of the outputs we use are taken after the animals have been humanely killed (to measure the accumulation of chemicals in the body). This is generally the least invasive set of procedures that can be done to give meaningful outputs to make scientific decisions about whether these chemicals are safe or not.

Why can’t you use animals that are less sentient?

The fish species used have been selected in accordance with the relevant Test Guidelines and the age ranges of the fish are such that they are of the least sentient that will allow evaluation of the specific outputs.

The species selected are representative of wild species. The data generated is therefore designed to protect these representative species in the environment thereby minimising larger scale environmental effects of tested chemicals.

Any fish that are showing a significant departure from the animal’s normal state of health or well-being will be identified and humanely killed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

When we identify that animals are showing adverse clinical signs, a “MONITOR CLOSELY” label is placed on their tank to make sure our technicians know this and increase their observations (all fish are observed at least once or twice daily), depending on the intensity of these signs. We have a list of potential adverse clinical signs within the licence which give examples of the type of things to look for that would suggest a fish is potentially unwell.

When we see that a fish is unwell, and is unlikely to recover, we humanely kill the fish to prevent and further suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

OECD Test Guideline 305 Bioaccumulation in Fish: Aqueous and Dietary Exposure.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

52. Translation of the immunological synapse

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunological Synapse, Tolerogenic dendritic cells, Autoimmunity, Cytotoxicity, Cancerimmunotherapy

Animals Types	Life Stages
Mice	neonate, juvenile, adult, pregnant, aged, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our research projects encompass immunological contexts ranging from tumour development (e.g. pancreatic cancer and breast cancer), infection (for example, with Listeria monocytogenes), autoimmune predisposition and induced inflammation (T-effector and T-regulator, and B cells). In all of these studies, our central objective is to further our understanding of molecular interactions that occur at the immunological synapse and their influence on the dynamics of interaction between immune cell types and on the overall immune response.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

We seek to understand and characterise interactions in infection, allergic inflammation, autoimmunity and cancer, situations in which the immune system plays a large role. This involves characterising such interactions at the level of cells, tissues and whole organisms with methods that allow us to follow interactions as they occur.

What outputs do you think you will see at the end of this project?

We are particularly interested in potential translational applications of modulating molecular interactions that occur at the immunological synapse. We will gain new information about immune functions on cell-cell and molecular level that will improve our understanding of their role. This data can be used in translational research to produce new approaches to treatment for autoimmune diseases or cancer, and can help to develop new medicines. All data will be published and presented at conferences and public science fairs.

Who or what will benefit from these outputs, and how?

There are a number of groups who will benefit from this research. In the short term, we will patent (when appropriate), share preliminary findings on pre-print archives and publish our completed work in peer- reviewed journals. Members of the scientific community will use our shared and peer-reviewed published research findings as inspiration for further innovation. Within the next 5 years, publication of our patents will enable licensing of patents by companies and potentially formation of new companies that will drive valuable economic activity in the UK and abroad. Work covered by previous patents has led to approved drugs being developed. In the longer term, beyond this license life time, patients with a variety of immunologically related diseases and diseases that can be treated through immunotherapy stand to benefit from products and best practices that are developed based on, or with input, from our research.

How will you look to maximise the outputs of this work?

We share all findings with the scientific community and are active collaborators with many research groups all over the globe. All data will be made available in preliminary form through sharing through open access archives such as Biorxiv, published in peer reviewed scientific journals (open access when possible), presented at conferences and public science fairs. Where appropriate, we will deposit datasets in open access repositories to allow other scientists to make use of the information. All other data referred to in peer-reviewed publications will be made available to qualified researchers on request.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have long served as the preferred species for biomedical research animal models due to their anatomical, physiological, and genetic similarity to humans. Advantages of rodents include their small size, ease of maintenance, short life cycle, and abundant genetic resources. Immune cells (T and B cells) in mice are mature at 6 to 10 weeks of age. This is the ideal age for immunological experiments.

Typically, what will be done to an animal used in your project?

Typical procedures are

Breeding of genetically altered animals (GAA) and wild type (wt) mice.

Irradiation of mice and transfer donor cells to investigate immune relevant cells. Mice will be kept up to 8 weeks after transfer.

Injections of substances: protein, lipids, infectious agents and small molecule biological modifiers, antigen presenting cells, and implantation of tumour cells. Mice will normally receive 1 to 3 substances, and a maximum of 8 substances may be injected in rare cases. Infections are cleared after a few days. Intra vital imaging: mice will be injected with a dye or tracer right before or during appropriate terminal anaesthesia. During the procedure, mice will be monitored, kept warm and fluids are provided. Surgical procedures necessary to expose the tissue of interest to the microscope will be done with aseptic techniques. The duration of the procedure is up to 6 hours.

None-schedule 1 methods of killing: required in cases when terminal anaesthesia is provided after intravital imaging whereby an overdose of general anaesthesia is administered followed by a schedule 1 method of confirmation.

Perfusion/ fixation: is used for tissue and organ harvest for histological samples to maintain full tissue integrity and cell morphologies.

What are the expected impacts and/or adverse effects for the animals during your project?

Irradiated animals will typically show signs of moderate ill health including reduced energy, partially hunched posture, diarrhoea and reduced water and food intake in the first two weeks after lethal irradiation. We expect that when irradiated mice lose more than 10% of their body weight (occurs in approximately 75% of the mice), they will begin to regain weight within 3 days. Mice are expected to gradually regain starting weight within 2 weeks after irradiation. Lethal irradiation followed by haematopoietic rescue has a treatment-related mortality of 2%, which is related to the transient cytopenia (including low platelets, red blood cells and white blood cells), which is associated with risk of bacterial infection.

In most mice (70%), partial ablation of bone marrow is expected to cause mild symptoms of malaise for up to 2 weeks. During this time, mice may be less active and show signs of un-groomed coat. In some cases (20%), reduction in food and water intake may occur, leading to maximum of 10% loss of their original body weight.

Pruritus may develop within days or several weeks following irradiation, leading to over grooming. This is expected to occur in up to 10% of the cases.

For administration of substances such as Antigens given in the form of peptides, protein/hapten complexes, or peptide-loaded dendritic cells are harmless and are expected to cause no extra adverse effects. Cytokines (e. g. Type-1 interferon), adjuvants, TLR agonists (e. g. polyIC), and microbial components (e. g. LPS) may cause systemic inflammation resulting in fever and very rarely shock. In known cases, the dosage and route of administration will be chosen to avoid systemic ill health.

Potential local infections or hypersensitivity either due to the administered substances or local wound from injection. Intra-peritoneal injections may very rarely cause peritonitis. Tumours mostly develops at the site of implantation by subcutaneous or intraperitoneal injection. Within the limits set for tumour growth (1.2cm diameter) and duration following injection (25 days), mice are generally in good health and no sign of cachexia or terminal disease are observed. Metastases are also rarely observed within these limits. Typical signs of ill health are piloerection, hunched posture, ulceration of the skin surface, and possible interference of the tumour mass with eating, ambulating, urinating or defecating.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Overall, we expect the following proportion of severities: 68.3% sub threshold, 2.7% mild, and 29% moderate.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The nature of studying immune cell communication requires consideration of the local environment in which the interactions take place (microenvironment). This complex mixture of factors supplied by various cell types within a tissue shape immune responses, making it essential to evaluate such hypotheses in whole body/tissue systems.

The complexity of the diseases of interest such as autoimmune diseases or cancer cannot be faithfully replicated without a whole-animal approach.

While systems involving self-propagating, modified cells in vitro exist, these behave in a substantially different, and less physiologically relevant, way to cells that could be sourced from genetically modified animals.

Which non-animal alternatives did you consider for use in this project?

For a significant part of our in vitro studies, we used ex vivo expanded human T cells instead of mouse derived cells. This allowed us to significantly reduce the total number of animals used.

To address molecular mechanism of extracellular vesicle formation we have developed tools to genetically modify human cells (using CRISPR-Cas9) to replace the use of knock-out mouse cells cancer models and therefore avoiding the unnecessary expansion of the mouse colony.

For the study of more unique cell populations, such as those found in the mouse lymph nodes, we replace them with cells derived from human tonsils and human splenic tissue that we get from a tissue biobank.

We are replacing administration of tamoxifen via an oral gavage by administering 4OHT to a culture of isolated cells, which eliminates additional stress on the animal by gavage.

Why were they not suitable?

These methods were suitable in some instances, but cannot replace the microenvironment of various cell types, their interactions and the ability to test effects of genetic alterations and substances in other instances. The available alternatives alone cannot answer the question of the effects on a whole body.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Number of mice per projects were calculated using tools such as literature search, discussion with other research groups that are in the same and or similar research fields, requirements of data points, experience and results of power calculations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used online tools such as NC3R's Experimental Design Assistant and similar software to calculate the required number of animals that will provide sufficient statistical power to address biological questions in combination with data from in vitro work. Based on our experience with particular disease models over the last 20 years we can also include the data from earlier cohorts to assist in the planning of group sizes where we can estimate the effect size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are using both genders for experiments, use of cells and tissues from shared control animals by multiple researchers, and reviewing mouse requirements regularly to avoid unnecessary breeding. Some of the models used to address our scientific questions do not follow Mendelian ratios and more breeding is required to obtain experimental animals. By examining the use of different genotypes in terms of the phenotype we have been able to identify suitable mice of a genotype that would previously have been deemed unsuitable. Where we have a need to address antigen specific components, we have generated mice with transgenic T cell receptor (TCR) and alleles predicted to alter lymphocyte function. By using lethal irradiation and bone marrow transplantation, we can reduce the number of animals bred as a single donor animal can reconstitute 8 lethally irradiated mice.

As a secondary mean to reduce the use of animals, whenever we need mouse T cells, we expand them up to 10-fold ex vivo. This significantly reduces the number of mice sacrificed for extraction of pure cell populations. We also freeze these cells and keep the records available to all lab members.

We generate antigen specific T cell clones and use ex vivo transfection or transduction of human T cell lines with specific TCR to replace as much as possible the use of TCR transgenic mice.

The strains we don’t need to use for any active project will be cryopreserved as soon as they are no needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild type and genetically altered animals. We breed mice for tissue harvest after mice are killed to investigate primary immune cells in vitro.

Mice injected with substances will be monitored for potential local infections or hypersensitivity either due to the administered substances or local wound from injection. Intra-peritoneal injections may very rarely cause peritonitis.

Animals will be exposed to irradiation to create an immune model that allow us to define effects of immune relevant genes and to determine quantitative contributions of different immune cells without resorting to complex breeding strategies. Mice will be irradiated with a maximum dosage of 1100 rads for bone marrow ablation, which will be typically split into two dosages of 550 rads each, separated by 4 hrs in order to induce total ablation of bone marrow. Irradiation will only be applied on adult mice.

Irradiated mice will receive haematopoietic precursors from different mice, which will be injected intravenous or intraperitoneal routes. Reconstitution will be performed within 24h post irradiation. Irradiated mice will not be subjected to other procedures until their full recovery.

For intra-vital imaging lasting for an hour or more the surgery will be carried out aseptically. All mice under a procedure will be monitored daily and will be killed immediately when any adverse manifestation such as ocular or nasal discharge, piloerection or hunched back appears.

Where the possibility of adverse effects is not known (e. g. administration of novel compounds), pilot studies involving small numbers of animals will be undertaken, starting with the smallest dose. The dose will be increased if innocuous and necessary.

Why can't you use animals that are less sentient?

Mice are the least sentient model that can mimic the mammalian whole body system.

We use adult mice that have mature immunological cells (T and B cells) that happens in mice between 6 to 10 weeks of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We import any strains of interest as sperm or embryo in case we can develop an in vitro experiment instead by the time the samples arrive and won't have to use them.

We consider the volume and route of injections, and where multiple routes are possible use the least painful and invasive route. We use of inhalation anaesthetic to reduce temporary discomfort during a procedure.

To tail vein bleed mice to collect blood for genotyping, we replaced the use of a scalpel with a needle. The needle puncture results in a smaller incision and therefore less stress for the mouse.

We will do pilot studies with primary cells in vitro for cancer models. We will use inducible conditional models that allow genetic modifications in specific cells at specific times, so the onset of disease phenotypes is highly controlled and limited to specific cell types.

To reduce the risk of infection in irradiated mice, antibiotics may be given in drinking water for 2 weeks post irradiation. Where possible, primary cells will be used in vitro instead.

We are picking up/ moving mice with the use of the tunnel in the cage when appropriate, e. g. setting up breeding, moving mice to different cages, or removing mice from the cages. We use of environmental enrichment and reducing social isolation of mice to a minimum by co-housing where possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3R's guidance and ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend conferences and meetings organised by NC3Rs and other organisations. New methods that don't require animals and non-animal replacement protocols can be found in the scientific literature and implemented immediately.

53. Control of worm infection by the immune response and drugs

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Helminth/worm, Anthelminthic, Mast cells, Inflammatory disease, Microbiome

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to understand the interaction between parasitic worms (nematode helminths) and immune cells (mast cells) and how this influences immune responses in disease. This can lead to a better understanding of how infections can alter immune responses to vaccines or in non- communicable diseases such as diabetes and arthritis and thus lead to the development of new medicines. We will carry out research into the basic mechanisms by which worms trigger or control inflammation and the role that mast cells play in this. It is also important to develop new drugs to control worm infections for both humans and livestock and thus we will also aim to evaluate new compounds for anthelmintic activity and assess how elements of the immune response contribute to their effectiveness.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Parasitic worms infect more than a quarter of the world's population and while they can be detrimental to health they may also play a role in protecting us from the development of autoimmune diseases and allergies. Autoimmune diseases affect more than 5% of the global population and costs greater than $100 billion while allergies affect more than 20% of the world with costs of $18 billion, with costs and incidence of both diseases increasing.

Understanding how worms interact with the immune response and vice versa is important to understand how inflammatory responses develop (an inflammatory response is when the immune system gets out of control and can result in allergies, autoimmune diseases, such as diabetes and rheumatoid arthritis, as well as cytokine storms like that seen in COVID infection). Therefore, worm infections have the possibility of altering the development of inflammatory diseases, asthma and cytokine storms.

Worm infections are also a considerable health and economic burden. More than a quarter of the world's population are infected with worms at a cost of €1.7 billion to the EU livestock industry. Further, there have been no new anthelminthics drugs on the market for over 20 years and resistance is increasing to those currently available. Therefore, the development of new anti-parasitic drugs using mouse models is essential.

What outputs do you think you will see at the end of this project?

The primary output will be to publish the findings in high ranking academic journals and at international conferences. This in turn will support applications for research funding and will provide employment for research scientists in the field.

The identification of novel anthelminthics would be of enormous potential benefit to human health, animal welfare and the economy.

The scientific information of the effect of the worms on the immune response is likely to be of interest to pre-clinical scientists interested in infectious, inflammatory and allergic immune responses.

The project will further our understanding of the underlying mechanisms involved in the expulsion of gastrointestinal helminths and the induction of inflammatory and allergic diseases thus informing rational design of novel therapeutic strategies for these diseases, all of which have enormous impact on the economy and the health care of humans. Experiments in this PPL using specific techniques such as imaging in live animals (IVIS) will provide novel data of the interaction of cells and parasites in the gastrointestinal system, and give additional insights of the role of mast cells in the induction, polarisation and amplification of allergic Th2 type responses. Studies using IVIS will also avoid the sacrifice of animals at various time points to study the biodistribution of reagents in organs, therefore reduce the number of animals used for research.

Who or what will benefit from these outputs, and how?

As this licence is for 3 years the long term outputs are a contribution to the body of knowledge in the area.

Infections with helminth remain a challenging health problem with global health, economic and societal impact. The new knowledge we learn from these studies will help us to understand the role of immune responses in controlling infection but also how they may affect the generation of inflammatory and allergic responses.

In the short term we anticipate identifying new therapeutic candidates for treatment of helminth infections which in the long term may lead to the development of new pharmaceutical products for clinical intervention of these diseases.

In the medium term the information is likely to be of interest to pre-clinical scientists interested in how immune responses are modulated by infection.

The new knowledge we learn from these studies will help us to understand the role of immune responses in controlling infection but also how they may affect the generation of inflammatory and allergic responses.

How will you look to maximise the outputs of this work?

I collaborate with colleagues within the University, in UK and internationally to promote the research in understanding the generation of immune responses associated with helminth infection and development of novel anthelminthics. The findings of our studies will be published in peer-reviewed journals for other researchers in the field, in addition we will disseminate our findings through presentations at national and international conferences, and thus there are ample opportunities to share data and results with the international community. We also aim to promote public understanding of immunology and helminth via our outreach activities.

Species and numbers of animals expected to be used

- Mice: 1100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Normal naive mice or genetically altered mice with modifications of specific genes or cells of research interest will be used in this study. Animal models for parasite infection have been the essential tools used in research in our understanding of the underlying protective and pathological immune responses accompanying helminth infection. Recent development of the understanding of these immune responses demonstrate that they closely resemble responses in humans. Mice are the lowest vertebrate group where models of parasitic infection have been developed and refined and give repeatable results of low variability and high quality. The models have also been refined to reduce suffering to the minimum levels whilst still giving satisfactory result. Only adult animals can only be used as the immune system of younger animals is not sufficiently mature to generate appropriate responses

Typically, what will be done to an animal used in your project?

Infection with gastrointestinal helminths is induced by the oral administration of infective larvae. The dose given has been adjusted to ensure that adverse effects are as low as possible without compromising the induction of protective immune responses. A typical experiment would compare genetically altered and normal naive and infected animals which are untreated or treated with drugs against the parasite or immunomodulators (compounds which alter the immune response) and examined at two time points. Assessments of these effects on parasite expulsion and development of protective and pathological immune responses will be carried out post mortem. Mice will then be monitored closely and weight will be recorded. Depending on the specific research aims, experiments often last 2 to 6 weeks, at the end of which mice will be culled and tissues analysed.

For evaluation of novel anthelminthics normal naive mice will be infected with the parasite followed by administration of novel anthelmintics which have been identified in in vitro studies. Mice will then be monitored closely and weight will be recorded.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effect for each genetic modified mouse colony will be dependent on specific genetic modification of the mice. The majority of mice have no gross phenotypic abnormalities, reproduce normally and mature to full age, just as wild type mice do. Some strains have mild phenotypes involving altered behaviour or activity, or susceptibility to disease due to immunomodulation. The information for the adverse effect for each colony will be obtained from the suppliers and/or from literature if published already. Other potential minor adverse effects associated with breeding and maintenance are: ear notching should involve only very slight and transient pain, and no healing problems. Haemorrhage after blood sampling is very rare and will be controlled by local pressure. Parasite infection may result in mild gastrointestinal discomfort for a maximum of three weeks (mild weight loss, slight hunched posture and mild piloerection), but it often lasts just a few days or even goes undetected. Moderate muscle discomfort may result (altered gait, reduced movement and abnormal posture) however these effects should be very rare. Possible adverse effects also include pneumonitis (shallow, fast breathing) from migration of larval stages of the parasite. This effect should be transient (< 48 hours) and rare.

Normally the adverse effects associated with imaging are not expected to result in any lasting harm. Possible adverse effects include: transient stress/discomfort associated with anaesthesia, plus dehydration and hypothermia. Repeated anaesthetic sessions may have a detrimental effect on the animals, but this will be minimized by using short-acting inhalation anaesthetics and making the imaging sessions as short as possible. Fluid support will be provided where appropriate. Mice will be kept warm throughout imaging sessions.

Potential adverse effects associated with dosing regimes of novel anthelminthics (e.g. different routes of injection) are not expected to cause any lasting harm, but may include distress or discomfort due to restraint as well as transient, momentary pain during injections. Some signs of discomfort e.g. listlessness, hunching, may be observed after injection of some of the agents to be used. This is normally mild and transitory (2-48 hours). Animals will be monitored regularly after injection and if any of the mentioned signs persist or deteriorate, humane end-points will be applied as described previously.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

The adverse effect for each genetic modified mouse colony will be dependent on specific genetic modification of the mice. The majority of mice have no gross phenotypic abnormalities, reproduce normally and mature to full age just as wild type mice do. The expected severity is mild (> 95%).

Mice infected with helminths are expected to develop clinical signs resembling human helminth infection, thus may reach a moderate severity (about 20%). However, control animals without infection will not reach such a level of severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The proposed studies investigate the underlying the mechanisms of generation of protective and pathological immune responses to worms. The complex interactions between the host and the parasite cannot be modelled in vitro and the parasites cannot be produced or maintained in vitro. These studies require an animal with an intact immune system and gastrointestinal system. The complex interplay of cells and cytokine (immune molecular messengers) interactions between the gut and immune organs in vivo simply cannot be replicated or replaced by any available in vitro models. It is absolutely critical that the functional studies are analysed in vivo. Mice are the best animals for this work because of their structural and physiological similarities to the human immune system. Mice are used as they are the lowest vertebrate group where models of parasite infection have been developed. There is no good alternative model than the use of mice that can answer the specific questions of the proposed project.

Which non-animal alternatives did you consider for use in this project?

Whenever possible we will use non-animal alternatives in this project. For example, in vitro studies will be conducted to initially inform the interaction between T cells, dendritic cells and mast cells and helminth infection. Cell cultures of mast cells with T cells with or without antigen presenting cells (APC) in the presence of helminth products will be conducted to assess the effects of cell activation and polarisation. However these in vitro studies cannot adequately model the complete and complex array of immune responses, such as the role of epithelial cells and the effects on the parasite, involved in the generation of protective and pathological responses.

We have previously developed in vitro culture system in the previous project licence and have successfully used this for the evaluation of anthelminthic activity. Although this allows us to identify candidate anthelminthics it does not evaluate their efficacy and safety. Therefore in order to achieve this we will then progress the most promising compounds to pilot in vivo studies before more extensive in vivo analysis is conducted.
We keep up-to-date with developments in the field (including non-animal alternatives) and check relevant databases on alternatives.

Why were they not suitable?

In vitro studies cannot adequately model the complete and complex array of immune responses involved in the generation of protective and pathological responses, particularly when assessing multi- system pathological responses.
For analysis of anthelminthic activity the parasite must be maintained in animals. In vivo studies are essential in determining activity of drugs since in vitro studies cannot assess the bioavailability of drugs or how they may be metabolised.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals to be used in each protocol below are projections based on current usage and potential research interests through collaborations. They are based on years' of experience with the models we use and regular consultations with our establishment's biostatistician.

Protocol 1: Breeding and maintenance of genetically modified mice, 600;
Animals bred under protocol 1 will move onto 3 & 4 as required.
Protocol 2: Maintenance of parasitic nematodes, 70;
Protocol 3: Assessment of anthelminthic activity, 180;
Protocol 4: Infection with gastrointestinal helminths, 250

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will take the following measures to ensure that minimum number of animals used in this project:
Use of minimum numbers in each experiment: The experimental designs and methods of analysis of the results have been discussed with the Statistical Services Unit at the University. Experiments will be designed to use the minimum number of animals per group consistent with obtaining data that can be tested statistically. These numbers are based on our considerable collective previous experience of the numbers required and power projection of numbers required for our future needs. For qualitative experiments, the amount of material required is the minimum necessary to provide an adequate description

(e.g. good quality histological samples). We have also planned to reduce the animal numbers by harvesting multiple organs for analysing. We will continually review our results to adjust the number of animals used in experimental groups so that we use the minimum number of animals to have statistically valid data.

Use of optical imaging (Protocol 4): Animals that have received bioreagents may be imaged to determine the biodistribution of reagents in the organs, and thus provide additional insights of the action mechanisms of the reagents in vivo. This will avoid the sacrifice of animals at various time points to study the biodistribution of reagents in organs, therefore significantly reduce the number of animals used for research.
NC3R's Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design-assistant-eda) will be employed to generate experimental workflows that lay out the design of core experiments. This will assist in planning, from group randomisation, through consideration and minimisation of nuisance variables in design/measurement/analysis stages, to the most appropriate statistical tests and required group sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use other measures to optimize/reduce the number of animals used in this project: We will work closely with staff at the animal unit for efficient breeding of mice in Protocol Small pilot studies will be performed for any new project to ensure the success of following in vivo experiments and reduce any unnecessary waste of animals. Following that power calculations will be performed to calculate the animal group size for subsequent experiments.

We plan to reduce the animal numbers by harvesting multiple organs for analysing and for isolation of infective larvae.

We will use non-invasive IVIS imaging to follow the course of infections and biodistribution of reagents over time in the same animals, obviating the need to cull animals at different time points.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the lowest vertebrate group where models of parasitic infection have been developed and refined and give repeatable results of low variability and high quality. The models have also been refined in previous project licences to reduce suffering to the minimum levels whilst still giving satisfactory results, by – for example - reducing the infective dose.

Why can't you use animals that are less sentient?

The project aims to investigate how protective immune responses are generated in parasitic nematode infection and develop potential therapies for patients and for livestock. The animal models used require the intact complexity of the immune and gastrointestinal systems in order to analyse the interactions between molecules, cells, tissues and organs in vivo which are important in the initiation, development and resolution of protective and pathological immune responses in helminth infection. The parasite can only develop to maturity in adult mammals, mice are the lowest group which can be infected and where effects on the immune responses can be analysed. Therefore, in vivo work using the proposed animal models is required.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use the following steps to refine our experimental procedures:
The experiments (all using mice) of helminth infection often last 4 weeks, from infection day to finish, although some will be up to 8 weeks or more to study the long term infections. All experiments will be kept to the minimum length needed for the experiment to acquire the necessary data.

We use an improved administration protocol (cleaning the injection site and using new needles, prior to administration of substances & carefully avoiding the leakage of injection reagents)

We use the least infective dose of parasites necessary to induce the necessary immune response whilst having the least adverse effects possible.
Mice are closely monitored after infection and supported by, for example, offering food treats such as baby food and extra nesting material.

The use of in vivo imaging systems (IVIS) will allow us to determine disease onset at earlier time points and thus terminate procedures before the disease reaches a more advanced stage.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?
Guidance from the NC3Rs of animals in research, LASA good practise guidelines and advice from RSPCA for laboratory animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will learn advances in the 3Rs through various opportunities such as the University annual 3Rs symposium, and information from NC3Rs and from our own NIO.
We aim to implement advances in the 3Rs in our research whenever we can. For example, using one needle per animal, handling animal by cupping rather than picking up by the tail and positive reinforcement (with treats) when using the IVIS. We also liaise closely with NACWO and NVS.

54. Defining immune mechanisms that promote inflammatory arthritis and associated co-morbidities to identify new therapeutic targets.

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Inflammation, Arthritis, Immunotherapy, Multimorbidities

Animal types	Life stages
Mice	pregnant, adult, aged, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to characterise the immunological mechanisms that promote joint inflammation in arthritis as well as co-existing inflammation in other tissues and to evaluate immune-modulating drugs as novel treatments for inflammatory arthritis and its co-morbidities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Rheumatoid arthritis represents the most common type of inflammatory arthritis and affects ~1% of the world's population. While the clinical management of rheumatoid arthritis has improved in recent years, it remains a disease with significant unmet clinical needs. There is no cure and approximately 40% of patients do not respond to current frontline therapies that aim to reduce joint inflammation (e.g., anti- TNF and anti-IL-6R therapies). Patients with inflammatory arthritis also experience severe co- morbidities (e.g., uveitis, cardiovascular disease, depression) that impact their quality of life and mortality. Ultimately, the proposed work aims to advance understanding of the immune mechanisms that promote inflammatory arthritis and its co-morbidities so that we can exploit this knowledge to improve the diagnosis and treatment of patients.

What outputs do you think you will see at the end of this project?

The data generated during the project will provide new information about the immunological relationship between inflammatory arthritis and its associated co-morbidities, including identifying the immune cells involved, their interactions and pathogenic behaviours within inflamed tissues. We aim to use this information to identify potential diagnostic biomarkers and to establish whether targeting specific immune mediators is effective in the treatment of arthritis and its co-morbidities. The outputs will be disseminated to the scientific community through presentations at national and international conferences and publication in peer-reviewed journals.

Who or what will benefit from these outputs, and how?

The ultimate long-term objective of the project is to identify treatments that will benefit patients with inflammatory arthritis. This will also reduce the socio-economic burden of the disease. Before this, the project will have short- and medium-term impact.

Immediate to short-term impact:

The data generated will address a knowledge gap that will benefit scientists working in the fields of immunology and rheumatology, and inform future studies. Investigators working on the project will gain skills in applying cutting-edge research methodologies to identify mechanisms that drive chronic disease. Publication of the outputs will enhance the research profile of the investigators, the group and the University.

Medium-term impact:

Our research aims to drive a step-change that leads to better patient treatment. Newly identified biomarkers and therapeutic targets that have the potential to improve patient care will be of benefit to pharmaceutical companies that have expertise in developing diagnostic markers and drugs for immunological diseases.

How will you look to maximise the outputs of this work?

During the project, research outputs will be disseminated via presentation at national and international scientific meetings (e.g., British Society of Immunology, American College of Rheumatology, British Society for Rheumatology, International Cytokine and Interferon Society). We also aim to publish the data in peer-reviewed international journals (e.g., Journal of Experimental Medicine, Nature Immunology, Arthritis and Rheumatology). To support future research, large datasets will be made freely available to the scientific community via public access repositories.

Established cross-disciplinary collaborations with fundamental and clinical scientists specialised in immunology, rheumatology and ophthalmology will provide an opportunity to clinically validate and translate our basic research outcomes. Our group collaborates with several industrial partners focused on developing novel immunotherapies. Thus, a framework is in place for our research outcomes to inform the design of clinical trials in patients with inflammatory arthritis.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the species with the lowest neurophysiological sensitivity suitable for these studies. Pre- clinical models of inflammatory arthritis in adult mice are well-established, highly reproducible and provide a proven platform to test and develop new drugs. Mice have been extensively characterised in immunological research and the reagents needed to track immune responses are readily available.

Typically, what will be done to an animal used in your project?

Mice will be used in studies to characterise the immune response during inflammatory arthritis. Upon arrival at the facility, mice will be allowed to acclimatise for one week. Mice will be administered substances that promote an inflammatory response resulting in arthritis. These will be mostly administered by injection under the skin or into the knee joint while under general anaesthesia. Mice will be monitored for clinical signs of arthritis. In some experiments, we will test potential therapeutic targets by administration of immune-modulating agents, typically via injection or by oral gavage. Some mice will have a small blood sample taken from a superficial vessel to track immune responses. Most experiments will last 1-2 months before mice are killed using a Schedule 1 method or by exsanguination or perfusion fixation performed under general anaesthesia and tissues recovered for analysis.

For experiments that will determine whether induction of inflammatory arthritis results in early signs of ocular inflammation, a small number of mice will undergo medical imaging techniques (e.g., optical coherence tomography) under general anaesthesia that will allow us to track structural changes in the eye.

To allow us to determine the roles of specific immune mediators involved in inflammatory arthritis, genetically altered mice will be bred and used in arthritis studies. These mice will typically be deficient in a gene, or genes, the regulate immune function. There are no adverse phenotypes associated with these genetically altered mice. As well as being used in models of inflammatory arthritis, mice generated under the breeding and maintenance protocol will be used for in vitro studies to understand cellular mechanisms that determine their functions.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be administered agents that will promote arthritis, resulting in joint inflammation and leading to cartilage and bone erosion. As a result, mice will experience joint swelling. During this time, they may show a slight loss of mobility and normal exploratory behaviour. The vast majority of mice will be used in a self-remitting and resolving model of arthritis in which joint swelling begins to resolve (after 2-3 days).

Some mice will be used in a model of chronic inflammatory arthritis, where they typically experience joint swelling for approximately 10 days before they are killed for tissue analysis. As far as possible, suffering will be minimised by the use of analgesia and close monitoring of the mice for any clinical signs of suffering (e.g., behaviour, piloerection, weight loss). Rarely, mice may experience an adverse response to reagents administered to promote arthritis, such as skin irritation at the site of injection. For all studies mice will be carefully monitored for unexpected adverse events and should these signs persist and not be ameliorated by mild veterinary intervention, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice (up to 2000 mice) will be used for breeding to maintain genetically altered lines and their tissues will be recovered for in vitro studies. These mice will therefore only experience the mild severity category.

Up to 1500 mice will be used in experimental protocols and are in a moderate severity category. Approximately 90% of mice used in these experiments will develop arthritis and the maximum severity of these studies is moderate. Approximately 10% will be used to track immune responses in the absence of arthritis, or before clinical signs of arthritis

appear and therefore experience mild severity. As far as possible, suffering will be minimised by the close monitoring of mice, use of analgesia and humane endpoints.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Reliable alternatives that can replace animals to study complex immune interactions during inflammatory arthritis, as well as disease interactions in co-morbidities such as arthritis-associated uveitis, are unavailable. This is because the complexity of a process that involves immune cell activation, cell interaction and communication, and cell movement from organs of the immune system to inflamed tissues, cannot be modelled in culture systems.

Which non-animal alternatives did you consider for use in this project?

Where appropriate, we use in vitro cultures to support our studies in mice, which can provide information about cellular mechanisms associated with the disease. For example, in understanding how individual cells respond to a specific inflammatory stimulus found in joint tissues. Studies involving human samples has also informed the clinical significance of the pathways we will investigate. While these approaches complement our in vivo studies, they cannot replace the use of experimental arthritis models in mice.

Why were they not suitable?

While cell culture systems can help understand cellular mechanisms associated with disease, they cannot replicate the complex microenvironment found within inflamed tissues such as the joint that are critical for disease progression. Many of the immune processes responsible for arthritis also occur before patients present with clinical symptoms that lead to a diagnosis. This early phase of arthritis is extremely difficult to track in humans. Current in vitro systems also cannot be used to investigate disease interactions (e.g., multi-morbidities such as arthritis-associated uveitis and cardiovascular disease) which is an important aspect of this project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Group sizes for experiments are based on data generated in our previous experiments (i.e., previously observed effect sizes) or a standardised effect size (e.g., Cohen's d of 1.5) for pilot studies where no previous data are available. Depending on the outcomes being measured, typically our experiments require between 6-10 mice per group. We estimate experimental usage at 300 mice per year. This is based upon the scientific objectives of our current grant-funded projects and grant applications currently being prepared and equates to approximately 10 experiments per year (with 2 independent repeats).

The number of animals used for breeding and maintenance is estimated at 400 animals per year. This is based on the number needed to maintain transgenic lines and supply genetically altered mice required for experiments. These mice will also provide tissues for in vitro studies that we will perform alongside in vivo models of arthritis.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure that experiments are sufficiently powered to identify biologically relevant changes while also avoiding using too many mice, we use online experimental design tools. Experiments will be designed using the NCR3s experimental design assistant (EDA) and its power calculator tool. We also consult the statistical software tool G*Power (gpower.hhu.de) when power calculations for more than 2 groups are needed, or where non-parametric analyses are used.

The use of serial clinical imaging (e.g., optical coherence tomography) will allow us to track clinical disease over time in individual mice. This reduces the number of endpoints where mice need to be killed for immunophenotyping, thus reducing the number of mice needed for the study. This also allows us to identify high-value timepoints for analysis (e.g., peak of inflammation, or resolution phase of inflammation) thus ensuring that mice are not subjected to procedures for analysis at less valuable time points. The use of omic technologies will also allow us to generate more information per experiment about the immune processes involved in inflammatory arthritis, which significantly reduces the number of mice used in studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding strategies, where possible breeding homozygous mice to reduce the number of animals required. Our genetically altered mice have been registered

with the University's Genetically Altered Animal Register and Tissue Sharing Network, which promotes sharing of tissues between research groups, thus maximising the information gained from research involving animals without increasing the number of animals used. Ad hoc tissue sharing between research groups has already resulted in novel observations and funding to pursue research into co-morbidities associated with arthritis. Mouse tissues from experiments are archived so that we can revisit the tissues to investigate new immune pathways that become of interest, as well as optimise new methods.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used as they provide an excellent model system to study inflammatory arthritis. The immune system of mice closely resembles that of humans and arthritis models have been established that closely replicate the human condition. Mice are also amenable to the genetic alterations needed for the study and numerous genetically altered lines deficient in immune genes of interest are already available. Reagents (e.g., antibodies) that can be used to test the therapeutic potential of blocking immune mediators are also widely available for mice.

While the procedures used in the study will themselves cause no more than mild transient pain or suffering, the majority of mice will go on to develop inflammatory arthritis as a consequence of these procedures, which is classed as moderate severity. Here, we preferentially use an arthritis model of lower severity. For example, >90% of mice that develop arthritis will be used in a model where mice develop self-remitting and resolving joint inflammation only in an injected knee, without the more progressive and systemic (affecting multiple joints throughout the body) joint involvement seen in other models. For this model, incidence is 100% with immediate and synchronous onset and a relatively short experimental time-course that minimises any lasting pain or suffering. Development of arthritis is monitored closely and does not impede the animals' ability to feed, drink and explore their environment. Some mice that develop arthritis (<10%) will be used in a more systemic poly-articular model of arthritis (i.e. affecting multiple joints). Here, the development of arthritis is closely monitored, does not impede the animals' ability to feed and drink, and mice are given analgesia at, or prior to, clinical onset.

Why can't you use animals that are less sentient?

Mice have the lowest neurophysiological sensitivity of the animals suitable for these studies. It is not possible to conduct this study in less sentient species as they either do not have an immune systems comparable to humans or the species specific reagents needed to undertaken the study are not available. Our studies are designed to gain insight into the immune mechanisms that promote inflammatory arthritis in humans and to test potential treatment approaches. Inflammatory arthritis predominantly affects adults and some children therefore it is not possible to investigate relevant mechanisms that promote the disease at more immature life stages.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures used to study the immune system and measure clinical signs of inflammatory arthritis (e.g., measuring joint diameters) themselves cause no more than mild transient pain or distress.

However, in mice with active arthritis there is potential for pain and discomfort associated with these procedures to be exacerbated. Where appropriate, this will be minimised by the use of general anaesthesia. We have introduced several further refinements based on published guidance and literature reviews. These include the use of tangle-free bedding to minimise arthritic limbs being caught, handling of mice with arthritis on soft surfaces (e.g., VetBed), the use of analgesia in systemic poly- articular arthritis models, providing environmental enrichment (e.g., tunnels, nest boxes, effortless access to food) for the animals, and tunnel or capture handling. All animals will be assessed daily for any signs of adverse effects. Monitoring regimes will be adapted as necessary to be proportionate to the anticipated or observed effects. For example, mice with clinical signs of inflammatory arthritis are monitored daily using welfare assessment scoresheets. Similarly, mice displaying any adverse effects will be closely monitored and the named veterinary surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) consulted where any advice may be needed. In the event that animals reach certain pre-defined clinical scores (e.g., joint swelling) or display unexpected adverse responses, they will be killed by a Schedule 1 method or other method specified in the protocol to avoid pain or discomfort.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

My group participates in regional 3Rs symposiums and 3Rs events to share and learn about approaches being implemented by other groups. The AWERB committee at my institute review current best practices and informs researchers on these by e-mail and newsletter.

As well as consulting online resources such as the NC3Rs website, we will keep up to date with published protocols in peer-reviewed journals that describe animal models of inflammatory arthritis, including recommended refinements. In this regard, our group has published methodologies in books that describe animal models for inflammatory arthritis

including considerations on how to improve experiments by implementation of 3Rs initiatives.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

My research group attends 3Rs events and the annual regional meeting organised by our NC3Rs coordinator, which provides a forum to learn about new 3Rs approaches used by invited external participants and other groups in the University. We will continue to stay informed by using the NC3Rs website and e-learning resources as well as consulting other resources available from LASA (Laboratory Animal Science Association) and FRAME (Fund for the Replacement of Animals in Medical Experiments) when appropriate. Postdoctoral Researchers and students within the group are also encouraged to join the early career researcher's 3Rs group to learn and share best practices

55. Development of vector control strategies and intervention methods concerning the spread of malaria

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mosquito, vector control, malaria, parasite, Anopheles

Animal types	Life stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

There is an urgent need to develop alternative control measures against the spread of malaria as well as other vector-borne diseases. By developing and applying our technologies, we hope any successes will lead to the provision of low-cost measures for vector control in countries where malaria is common, on a continent-wide scale.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vector-borne diseases are a growing threat to public health in many countries, a development that may be compounded by climate change and invasive vector species such as Aedes albopictus. This is also reflected by the current worldwide malaria situation

as malaria is still one of the world's most devastating diseases. Malaria is an infectious disease transmitted by female Anopheles mosquitos caused by a small parasite of the genus Plasmodium. 200-400 million people are infected with human malaria per year, making it one of the most important diseases in the world with half of the world's population still at risk. It claims half a million deaths per year, with children below the age of five being the primary victims. These figures underline the importance of gaining in-depth knowledge of the disease to come up with effective countermeasures such as a vaccine, drug or vector control measures.

These tools are based on altering mosquito genes to determine how effective these genetic changes spread through mosquito populations as well as how any resulting offspring are affected. Female mosquitoes have the ability to bite and feed on human blood, therefore our main focus is genes that affect fertility and female biting capabilities in order to reduce the number of disease-carrying vectors in the wild that can infect humans, thus ultimately reducing malaria.

What outputs do you think you will see at the end of this project?

In regard to the project, my group’s main focus is to contribute towards new approaches to vector control in a bid to eradicate malaria.

The main mode for vector control is currently the use of indoor residual spraying (IRS) and physical barriers. Unfortunately, these tools are limited and often result in mosquito populations developing resistance. To provide efforts in expanding potential solutions, this project aims to continuously identify and test elements involved in determining the sex of mosquitoes as well as critical genes associated with mosquito physiology. This will provide better insight into basic mosquito biology.

In doing so, we also aim to continue generating genetically modified mosquitoes as well as preserving our current stock. Various mosquito lines with different genes will be tested and evaluated in small, caged experiments by my team in specialised enclosed facilities to assess behavioural changes and their ability to continue spreading malaria, for which we hope to see a reduction.

With all this, I hope to continue exhibiting promising research through public forums and scientific publications that illustrate new findings. Any positive outcomes are often shared with our collaborators for further studies.

Who or what will benefit from these outputs, and how?

With 3.4 billion individuals, up to half the population, at risk of malarial infection, there is a pressing need to wipe out this disease. Current strategies used include diagnostic management, drug discoveries and surveillance or contact control with parasitic carriers. This research is part of a large research consortium with any technologies that stem from our work being made publicly available.

In regard to my project, my main focus is to contribute towards new approaches of vector control as well as furthering our understanding of key features of mosquito biology essential for its strong success as a vector of diseases. Our short-term goals are to continuously identify key mosquito components in addition to manipulating these elements. Resulting genetically altered (GA) mosquitoes will then be tested in caged experiments and assessed to identify potential strains that; 1) deviate from the behavioural norm; 2) have altered reproductive capabilities; and 3) potentially lack the ability to carry or transmit the malaria parasite. We also aim to develop and test methods that identify the occurrence of resistance in our gene-drive systems (promotes biased inheritance to produce a population of mosquitoes with a particular genetic make-up) as well as ways to minimise this.

The study of malaria as a model for infectious vector-borne disease has revealed the relationship between parasites and their hosts as well as furthering the understanding of how genetics and biology reflect the behaviours of these carriers. For example, as a consequence of understanding the life cycle of the parasite within different hosts, other researchers are able to elucidate new drug target sites which could translate into prospective new treatments.

In the long-term, promising results can be expanded by our collaborators in endemic countries whereby small subsets of modified mosquitoes are released in a controlled manner and are subsequently evaluated in the field. These are major steps towards eliminating the spread of malaria.

As a result, my research is largely shared amongst scientists and fellow researchers via conferences, publications and collaborations. This aids the expansion of novel control methods in endemic countries through technologies that rely on interfering with typical mosquito behaviour or the infectious relationship of the parasite within the insect.

Overall, successes in this project could lead to improvements in vector control for the general public, and thus the spread of malaria, on a global scale.

How will you look to maximise the outputs of this work?

My funding and scientific investigations are largely based within the network of multiple institutions and researchers from across 3 continents where we are able to develop innovative ideas and disclose potentially world-changing research. My work is often published in open-access journals.

Many of our smaller cage trials are expanded to larger populations and therefore, my group frequently collaborate with universities and specialised centres across the globe. On top of my expertise, the sharing of data, transgenic strains and state-of-the-art research facilities in addition to frequent forum updates and conferences enable me to greatly increase the chances of successfully meeting my objectives.

Under our funder's mission statement, we also aim to continually improve community awareness at home in the UK as well as abroad in sub-Saharan Africa where my research will directly affect, or at the very least, involve these communities.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There are over 100 species of Plasmodium, a malaria-causing parasite, with 5 that have the ability to infect humans and many others that infect other animals, thus are considered to be zoonotic (i.e. can transfer between animals and humans) or species-specific malaria.

Plasmodium berghei is a strain of the parasite in rodents that has been deemed suitable for malarial studies using rodents as appropriate models of disease. Genetic processes and crucial molecules required for parasitic invasion are largely preserved. Here, we can study the underlying mechanisms and interactions between the host (mice) and parasite.

P. berghei is safe and easy to handle at all developmental stages and requires a live rodent host to establish and increase levels of the parasite. Previous investigations into culturing the parasite through non-animal alternatives (in vitro) have proved to yield unsatisfactory results; including limiting the parasitic lifecycle stages required for the project. Furthermore, a regular supply of infected blood is required for the mosquitoes to feed on. Ultimately, mice were selected as our chosen least-sentient rodent species.

As mentioned, the establishment of new mosquito lines as well as the maintenance of our current, more difficult strains will need to be maintained through regular delivery of blood meals. Though we are currently utilising new methods to reduce the number of mice, this blood feeding is best done on live animals due to characteristic mosquito behaviours.

Additionally, as a result of biological and physiological changes that occur with age in rodents, we have selected to use adult mice to reduce any experimental impact such variables would introduce.

According to Jackson et al. 2017, key immunological markers signify various developmental stages within mice, and it is understood that neonates do not possess an established immune system or the bodily/ blood composition required to safely and successfully propagate malarial infections within the first few days without potentially developing severe reactions. Due to the fragility of their systems, this would place unnecessary biochemical stress on young mice.

Additionally, mice nearing senescence may also respond differently.

Typically, what will be done to an animal used in your project?

The entire process for general blood feedings can take between approx. 30-90minutes, depending on the number of cages needed to be fed in relation to the number of mice under general anaesthesia.

Here, one mouse can feed multiple cages. The animals are initially weighed before the correct dosage of anaesthesia is administered via an injection in the abdomen cavity (IP). Once anaesthetised, the mice are placed on top of mosquito cages with solely the abdomen being exposed, ensuring more sensitive areas such as limbs, face and genitals are protected from mosquito penetration. Once mosquitoes have been adequately fed, mice are then humanely killed.

For infection of the malarial parasite by IP injection, animals are infected for approx. 3-5 days and monitored daily including regular weighing. Blood samples from superficial blood vessels, tail smears or punctures are routinely taken every 2-3 days to observe the levels of the parasite within the blood under a microscope.

Once sufficient parasite levels are achieved, mice are put under general anaesthesia and blood is either collected via cardiac puncturing (directly from the heart) and exsanguination (draining of blood) or mosquitos blood-feed on the infected mice. This is followed by the humane culling of the animal to reduce unnecessary suffering.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected for mice used solely for mosquito blood feeding as the animals will be anaesthetised for the duration of the procedure and then humanely killed.

All animals regardless of their infection status will be checked daily. The weight of the animals will be recorded on the day of infection and as mice are only infected for a few days before being anaesthetised for blood collection or mosquito feeding, clinical signs of malaria should not occur for the duration of this project.

Regardless, very few animals (<1%) can suddenly die from malaria infection. Therefore, any of the following reactions require symptoms to be reduced with some form of treatment and monitored 24- hours prior to killing via a Schedule 1 method if there is no improvement: piloerection, pallor, shivering, hunched appearance, reduced activity, elevated heart rate or distress.

More specific clinical signs of malaria include, but not limited to, respiratory distress, reduction in mobility or 15% loss of body weight compared to the day of infection. Neurological symptoms include convulsions, reduction in coordination and, worst case

scenario, paralysis followed by death. Any one of these symptoms and the mice will be killed by a permitted method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)

The expected severities that mice used in our project will experience will vary. Approx. 30% will be non- recovery, 50% of mice will experience a mild severity and the remaining 20% will experience a moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present, we require the use of animals in order to meet the objectives of my research. Maintenance of mosquito colonies require regular provisions of a blood meal for egg production, and this is best done on live animals due to mosquito behavioural biology. Direct feeding on humans poses a health and safety risk and therefore, mice are required for some procedures such as the establishment of new GA mosquito strains, which require high feeding rates and a large number of progenies, or simply for malarial infection of mosquitoes through blood meals.

Because genetic processes and essential molecules of the invasion strategy are largely preserved among mammalian parasites, we will continue exploiting the parasite, Plasmodium berghei, as a model for malarial infections, a safer alternative to the human malaria, P. falciparum. Additionally, the culturing of specific-life stages of the parasite in vitro (using non-animal alternatives) has yielded unsatisfactory results while the regular supply of fresh red blood cells present in live rodents allows for sufficient natural replication and subsequent infection of mosquitos that require blood meals. Conversely, initially cultivating the parasite in vivo (in mice) can help with culturing it in vitro (i.e. petri dishes) for short periods of time to perform specific experiments.

Which non-animal alternatives did you consider for use in this project?

We have largely reduced the number of animals we expect to use due to the measures put in place to replace animal feedings during our previous project. Where possible, we had initially moved from maintaining our large stock of mosquito colonies with mice to

membrane feeding using donated human blood. We have more recently moved towards using cow blood as a more sustainable and effective option.

We have also taken to experimental modelling to identify the nature of some of our GA lines under various conditions. Statistical and genome-wide transcriptional approaches allow us to characterise these mosquitoes all the while reducing the need for larger population experiments, and consequently the need for live animals.

Why were they not suitable?

Unfortunately, generating new mosquito strains with altered genes can prove difficult. When moved to membrane feeding, a number exhibited a fitness cost, meaning typical mosquito behaviours such as feeding, egg production and fertility rates were affected.

Additionally, parasites grown in vitro show altered genetic patterns related to factors produced by disease-causing organisms, metabolism and reproduction, in turn affecting any follow-up experiments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our main aim is to establish and maintain mosquito lines in addition to qualitatively assess the function and limitations of these lines via malaria infection and observation. Due to the replacement measures that have been inaugurated within our lab, i.e. artificial hemotek feedings, the number of mice estimated reflects the numbers required to meet our scientific objectives.

This is largely determined by the number of lines that need to be generated throughout the duration of the project licence which is largely based on my projects in previous years and will also correlate with any additional funding my research may receive.

Furthermore, the number of mice required is dependent on our findings from our infectious experiments including that stated in protocol 2 and thus, there is the possibility we may need to further investigate our results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have consulted with the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO) in many instances concerning reducing the numbers of

animals in past licences. We have reviewed our experimental procedures as well as the actual animal work performed under the current and past projects. The number of animals proposed to be used under this licence will be reduced by continuing to implement the use of membrane feeding over more GA strains, including newly generated lines.

We will continue to fully transfer mosquito colonies to membrane feeding so that mice are only used in cases where membrane feeding proves prohibitively inefficient. We will also continue to reduce the number of mice used by using the whole mouse blood to maximise the number of mosquitos that can be fed per animal as well as replenishing our stock of the parasite.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to abide by the NC3Rs in addition to any updates or advice.

When possible, we aim to preserve the parasite by freezing blood obtained via cardiac puncturing to avoid animal wastage as the parasite strains do not then need to be maintained by continuous breeding.

As mentioned, we will also continue to use the whole mouse blood to maximise the number of mosquitos that can be fed per mouse as well as utilising transcriptomics (a terminology for the study of active genetic sequences which can help give us a broad view on which processes in the cell are active, and which are dormant) and statistical modelling where appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are commonly used as models for human diseases to provide insight into these mechanisms, providing validity and reproducibility.

Animal suffering is minimised by conducting blood feedings of mice to mosquitoes under terminal anaesthetics, thus culling is the end-point in both protocols. During this time the depth of anaesthesia is monitored by pinching the toes, observing any reflexes and re-administering additional drugs where appropriate.

To minimise suffering, injections and blood samplings are carried out under sterile conditions by competent scientists and mice are then monitored daily for any signs of

suffering or distress. Infected blood is either obtained through exsanguination or used to feed and infect mosquitoes whilst mice are under terminal general anaesthesia, to reduce pain and suffering, before culling.

Why can’t you use animals that are less sentient?

Mice have been selected due to being the least sentient species that still exhibit similar responses that provide a proof of concept in our field of study when observing malarial infections within humans and rodents. All mice used for mosquito feedings will be terminally anaesthetised and humanely killed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As mentioned, we have previously made several refinements to the protocols to ensure minimal pain and distress during procedures. Animals will be monitored daily for signs of any adverse effects and aseptic techniques will be used to minimise the potential for infection. Animals showing signs of adverse effects will be killed using humane methods to minimise suffering and where appropriate, investigated and relieved under veterinary advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We continue to follow ASPA, NC3Rs and LASA guidelines to ensure experiments are conducted in the most refined way possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We aim to continue keeping in contact with animal technicians regarding any notices or changes. We will continue to discuss current and new approaches to procedures, opportunities and ultimately the welfare of our animals with our network of NACWOs, veterinarians and animal technicians. Where appropriate, nominated PIL holders will attend NC3R’s workshops, webinars and courses throughout the course of this project.

56. Novel drugs for intractable cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Drug discovery, G protein-coupled receptor

Animal types	Life stages
Mice	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We wish to develop new chemicals, antibodies and other drugs that slow, stop or reverse the growth of cancers that currently have no or few life-extending treatments. Our principal targets are receptors for hormones that provide benefits for cancer cells to grow and spread around the body. We make chemical compounds and antibodies that are very selective - that is they target specific hormones or subsets of receptors for a specific hormone to provide benefits with minimal side effects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The outlook for most cancer patients has improved dramatically in the last 30 years, and mean survival after diagnosis is around 10 years. However, these figures disguise a marked polarisation of treatment efficacy. Some cancers have just as poor outlook as they did 30 years ago and there are no treatments that extend life for a significant time. Our targets include pancreatic cancer, a devastating disease that usually results in a terminal prognosis at first diagnosis and 1-year survival of under 25%. We also hope to address cancers that are responsive to treatment but become mutated at which time they have a short terminal course. Such cancers include "hormone refractory" prostate cancers that lose their response to anti-testosterone drugs. Other targets include "triple negative" breast cancers which do not respond to drugs that save the lives of the majority of breast cancers patients. Finally, we wish to target rare cancers. One example is a very rare lymphoma (Sézary syndrome) whose 5-year survival is under 20% and in which the only effective treatment works in just 28% of patients and extends life by only around 7 months. While some of the patients in these cancer diseases are older, there is significant representation among the working-age population.

What outputs do you think you will see at the end of this project?

Outputs from this work will include information in the form of new knowledge, which may be published in the scientific literature and disseminated at learned society meetings. We are focused on translational research, so patent filings may be made prior to publication and they are outputs with tangible value for the development of novel therapeutics. Naturally, drug development requires both significant time and funding, but our programmes have developed and the University has approved formation of a spinout company to progress its activities. Outputs may also include the development of new drugs - for example, our current research programme has resulted in a clinical candidate.

Who or what will benefit from these outputs, and how?

Publications benefit staff career development and increasing the sum of knowledge in the public domain. That usually leads to academic contacts, collaborations and further advances with their own outputs.

Patents provide the opportunity for novel inventive science to be developed by biotech and pharma companies with a route to recover research costs through the sole rights to manufacture and sell drugs during patent life. This provides commercial benefit to the companies, with revenue shares flowing back to supporters of earlier research. Supporters in this case include funders - where income supports new science - and the University, where income not only provides for growth of academic activities but also increases the institutional reputation within the national and international communities.

Patients will benefit from our novel efficacious therapeutics with extension of life and improved quality of life. Our approach is to target disease precisely so that drug effects are tailored to the appropriate population of patients who should receive benefits without the level of toxic side-effects experienced with less selective cytotoxic therapies. Even if a cure is not possible, acceptable life-extending therapies have great value for some cancers that are currently very intractable to treatment.

The families of patients with intractable diseases have to come to terms with their relatives' illnesses and in some cases the brevity of life after diagnosis can make this a very traumatic experience.

Increased lifespan can allow more time for families to come to terms with what are still ultimately fatal diagnoses.

Healthcare providers (NHS in the UK and equivalent state healthcare providers in other countries, insurers in other countries without state provision of medicine) would benefit from reduced costs for treatment and palliation of protracted disease and reduced pressure on bed space. Even medicine that do not extend life greatly, but allow for acceptable quality of life in the home setting reduce healthcare costs and improve patients experience.

The national economy would benefit from UK drug discovery processes through employment, taxation and would also gain international recognition for the quality of UK science .

How will you look to maximise the outputs of this work?

Our experience in taking a research programme from academic basic science through drug discovery research and into a spinout company seeking major investment has been a very educational experience from the start. The interactions with medicinal and synthetic chemists, molecular designers, clinical development experts and commercialisation professionals has given me insights into the process from basic science to the clinic that has influenced my outlook greatly. I understand the need for professional industry-standard data packages, and in this context, our experiences with the translational aspects of drug development beyond efficacy models (PK, DRF and MTD studies) has been transformational. One specific issue may appear counter-intuitive, but it is clear that publication too early can corrode the value of IP, so we do not rush into dissemination of information into the public domain without proper protection as that would reduce the likelihood of successful development of a clinical therapeutic.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice represent the appropriate mammalian species for these studies. Commercial development of novel agents is unlikely if an industry standard data package (i.e. one using mouse models) does not support mammalian efficacy. (We are not proposing to work to GLP standards for discovery research, but to use GLP-like standards which will aid later translational and commercialisation activities).

Mouse models are also well developed, characterised and refined.

Typically, what will be done to an animal used in your project?

During the studies, animals will receive vehicle or drug agent treatment, usually by injection (but possibly by addition to the water) so that injection constitutes a relatively minor harm in these studies. Studies will generally last no longer than 28 days after the start of treatment. In most cases tumours establish and can be treated within a week of implantation of cells.

Subcutaneous xenograft models

Inhalation anaesthesia, subcutaneous injection of cells, measurement of tumour sizes with vernier callipers. (Optional IVIS visualisation, if appropriate)

Orthotopic pancreatic xenografts

Inhalation anaesthesia, laparotomy, intrapancreatic cell injection, wound closure, up to twice weekly inhalation anaesthesia for IVIS visualisation.

Orthotopic breast cancer

Inhalation anaesthesia for cell injection into the mammary fat pad, measurement of tumour sizes with vernier callipers. (Optional IVIS visualisation if appropriate)

Systemic injection of lung tumour cells

Intravenous injection of lung tumour cells to the tail vein or by intrathoracic injection under brief general anaesthesia, up to twice weekly inhalation anaesthesia for IVIS visualisation.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts and/or adverse effects

Effects due to tumour presence - subcutaneous models: ulceration, orthotopic models: pressure on local organs or tissues causing pain or interference with normal function (e.g. gut transit for pancreatic cancer model).

General health adverse effects:

- Weight loss of 10 - 15% of the animal's average stable weight
- Hunched posture
- Piloerection
- Altered behaviour/activity, which may include abnormal locomotor activity, under-activity, hyperactivity, under-grooming, over-grooming, scratching, licking, or self-mutilating
- Dehydration
- Abnormal vocalisation
- Persistent hypothermia
- Enlarged lymph glands
- Anaemia
- Eating/drinking difficulties
- Mild dyspnoea

If any one of these endpoints is severe or prolonged (>24 hours), the animal will be culled immediately.

Mice will be humanely killed, by Schedule 1 method, in the unlikely event that they show any one of the following signs:

- Body condition score ≤2/5
- Weight loss of >15% of the average stable weight, if not improved within 24 hours
- Internal tumour growth begins impeding normal function
- Serious clinical signs (e.g. incontinence, diarrhoea, >mild dyspnoea, bleeding/mucopurulent discharge from any orifice, hindlimb paralysis/weakness, tremors or convulsions)
- Pain which cannot be promptly relieved
- Hind limb paralysis/weakness/'flippering' gait
- Signs of being moribund (lethargic, failure to respond to gentle stimuli) Duration

We expect adverse effects to be short-lived as we will euthanase any mice experiencing significant suffering. We expect the incidence of adverse effects are also likely to occur towards the end of the experimental period as tumour sizes increase.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We propose that all the studies will be of moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

During the process of development of a new chemical compound, we first test in computer models (in silico) with protein models that mimic the structure of our target hormone receptor. Then we test in cells (in vitro) that express the target receptor, by measuring the effect of compounds on hormone signaling to induce changes in growth and proliferation of cancer cells, as well as their ability to migrate and invade new tissues. Once we have exhausted the range of tests in cells (in vitro), we must measure the effects of potential new drug compounds on tumours in a living organism (in vivo). This means implanting tumour cells into mice so that we can measure the growth differences between in groups treated with drug candidate molecules compared with groups given the same vehicle solutions without the drug compound. It is currently impossible to replicate the complex interplay of effects that act on tumour cells in a living organism within a 2D/3D cell culture, so the studies we propose are an essential for development of new drugs. Additionally, animal models are currently an industry standard for a data package to be used to support preclinical development and clinical trials in humans.

Which non-animal alternatives did you consider for use in this project?

In addition to considering alternatives, we do in fact use them in the earlier stages of drug discovery.

We model our receptor target using sophisticated computer methods, and design drug molecules to fit the structure. We then virtually screen these molecules to determine which are likely to bind to the target receptor. Thereafter, we synthesise and test the selected compounds in in vitro cell models in all cases to measure the effect of compounds on signalling pathways and specific cancer hallmarks (proliferation, apoptosis, migration, invasion etc.) before performing animal studies.

Why were they not suitable?

They are suitable to identify compounds that block the receptor and to determine the nature and mechanism of those effects, in the early stages of drug discovery. However, they lack the complexity to allow us to determine their likely effect in humans, so we must move on to animal studies when all these other tests have been performed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We know the group sizes for the studies we expect to perform and those have been chosen to be the minimum to provide robust statistically relevant results. Depending on the minimum detectable effect and availability of historical data, group sizes are typically 8-10 mice. Within the oncology community, there is wide use of relevant animal models. We do not originate them, but use those which are recognised as well as characterised, refined and ethically acceptable in numbers that lead to identification of changes of magnitudes that are scientifically and clinically relevant.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We take advice from the local statistical professionals to design experiments that use the smallest number of mice appropriate. We use groups of mice that are very closely or identically matched in age, sex genetic strain and size, because that reduces the spread of results. As we generate more data, we will also incorporate the use of NC3R's Experimental Design Assistant to determine sample size calculations for future experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to consult statistical professionals and to keep abreast of the scientific literature relating to use of mice in order to benefit from advances that lead to reductions in numbers. We incorporate many non-animal methods in our drug development pipeline such as in silico screening (docking/binding), in vitro screening (potency/selectivity), in vitro cancer models (proliferation, apoptosis, migration, invasion, angiogenesis and more), in vitro ADME and pharmacokinetic studies. From these methods, we select only a handful of lead compounds to test in mice from a long list of more than 1000 compounds.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use several different types of cancer models (Workman et al Br. J. Cancer 2010).

Subcutaneous xenografts

Human cells are implanted under the skin of the flank (side of the body) of mice so that they grow into tumours. The mice would normally reject human cells so we use natural or artificial mutant mice that lack a complete immune system so that they tolerate non-native cells. Those mice are reared and kept in very clean conditions to prevent infections (because of their reduced immune status) and all our experiments are performed in those clean conditions with staff wearing gowns, gloves and masks to protect the mice. The procedures are not painful, as the implantation of the cells is an injection performed at the loose skin of the flank after carefully restraining the mouse. Short term anaesthesia will be used if it is required to minimise duress and duration of procedure. The most likely reason for this is that some mice are temperamentally less calm about handling and injection, so it is more humane to anaesthetise them to reduce the stress experienced. As the tumours grow, they do not cause pain, and if the size reaches a limit specified in the protocol, the mice are euthanased.

Orthotopic xenografts

Here, the cells are implanted into the tissue or organ in the mice in which the human cancer originated. For example, human breast cancer cells are implanted into the mammary tissues of the mouse. Where the tumours can be assessed by looking at the mice, we measure their size and can assess their progress towards an end-point size limit or whether there is any pain, suffering or distress. If cells are implanted into internal organs, the tumour cells may be altered genetically so that we can see them through the body wall, by virtue of their ability to emit light under specific conditions (e.g. luminescence, fluorescence). This allows us to assess size of tumours within the body.

Syngeneic orthotopic models

In these studies, we inject mouse cells into the tissue of origin of the tumour. This allows us to assess tumour growth in mice with a complete immune system, which is a more realistic model than the studies in immunodeficient mice. These models may provide closer approximations of cancer in that they can spread to other parts of the body, so observation of the mice, assessment of tumour sizes (and any secondary tumours) is even more thorough and signs of distress are watched for very carefully.

For all these studies, at the end of the experiments mice are all euthanased painlessly. We also keep a close check on the mice with a team including vets, and highly experienced animal care workers who can tell if mice are generally unwell or showing signs of any distress.

Why can’t you use animals that are less sentient?

We need to use mammals to provide systems that work in the same general ways as in humans. Mice are the least sentient mammalian species that we can use. While non-mammalian models for in vivo research in cancer biology are available (e.g. zebrafish models), they are not widely accepted in the same way as mammalian models in their perceived power to predict human responses. Therefore, to aid translation/commercialisation of our research into practice and eventually patient benefit, we propose that mouse models are the most appropriate ones.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We perform studies for the shortest time to provide robust data. We choose models and techniques that cause the minimum of harm discomfort or suffering. Pain will be controlled during surgery by general anaesthesia and post surgery by analgesics. Surgical sites will monitored daily for signs of inflammation and infection. In the event of post-operative complications, mice will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions. If an orthotopic tumour’s presence is associated with signs of pain or suffering we would consult the NACWO and/or NVS for advice over continuation and/or analgesic treatment, and euthanase mice if those signs are maintained for more than 24 hours.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website contains a vast range of guidance for best practice across the spectrum of animal research activities. (https://www.nc3rs.org.uk/3rs-resources). CAAT-Europe also disseminates information about developments within 3Rs in the European network, which NC3Rs is part of.

PREPARE guidelines (produced by a group of experts from the UK and Norway) for planning animal experiments is also used as a guide to plan individual experiments. All surgeries will be performed in line with the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We attend scientific meetings and relevant training courses in the UK and abroad. We read the scientific literature. We discuss the 3Rs with staff in the animal facility. We Also consult our NC3Rs regional programme manager. Where advances are made, it is ethically indefensible not to implement them.

57. Establishing the pathophysiological impact of cigarette smoke extract in mice as a model for chronic obstructive pulmonary disease (copd)

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Cigarette smoke extract, Chronic Obstructive Pulmonary Disease, Underlying mechanisms, Disease progression

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To establish a mouse model of cigarette smoke extract-induced Chronic Obstructive Pulmonary Disease (COPD) with which to determine the underlying pathophysiological changes to health.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic Obstructive Pulmonary Disease (COPD) represents a significant burden to public health with an estimated cost to the NHS of £800m. The hallmark of COPD is chronic inflammation that affects central and peripheral airways, lung tissue and pulmonary

vasculature. Repeated injury and repair leads to structural and physiologic changes. The inflammatory and structural changes in the lung increase with disease severity. The main components of these changes are narrowing and remodelling of airways, increased number of goblet cells, enlargement of mucus-secreting glands of the central airways, alveolar loss and finally, vascular bed changes leading to pulmonary hypertension. Evidence suggests that the host response to inhaled stimuli generates the inflammatory reaction responsible for the pathophysiological changes. Activated macrophages, neutrophils, and leukocytes are the core cells in this process. Oxidative stress and an excess of proteases amplify the effects of chronic inflammation. Hyperinflation and destruction of lung parenchyma predispose patients with COPD to hypoxia.

Progressive hypoxia causes vascular smooth muscle thickening with subsequent pulmonary hypertension, which is a late development conveying a poor prognosis.

Current COPD therapies are largely inadequate due to their modest improvements on symptoms and long-term mortality. Therefore, the development of better models for COPD with which to develop new therapies is essential. In mice, many of the hallmark features of human COPD, namely (i) lung inflammation, (ii) impaired lung function; (iii) emphysema; (iv) mucus hypersecretion and (v) airway and vascular remodelling can be induced in response to cigarette smoke. However, while rodent cigarette smoke extract models of COPD exist, their design and ability to address the underlying pathophysiological mechanisms are limited for several reasons. First, the majority of studies assess the impact of COPD in only a single tissue, typically the lung, failing to acknowledge the effects of this disease in other tissue types or on a systemic scale. Indeed, systemic inflammatory mediators may contribute to skeletal muscle deterioration, and initiate or worsen cardiac, metabolic, and skeletal comorbidities. Indeed, comorbidities are frequently reported in COPD patients, and it is important to understand whether or not the onset of these comorbid conditions are as a result of immuno-pathogenic mechanisms of COPD in additional tissues. Therefore, new models which adopt a whole body, multiple organ/tissue analysis approach are needed in order to gain new insights into how the disease progresses, whether tissues separate to the lungs are also predisposed to developing additional pathogenic phenotypes and the role of the peripheral immune system in driving them.

Additionally, existing studies typically only focus on the impacts of COPD in a single sex. In humans, studies have shown that the impacts of COPD are different in men than women, with women typically presenting disease characteristics sooner than men and at a more severe level. As such, understanding how the disease develops and progresses in both sexes is critical for developing new sex-specific therapies for men and women.

Furthermore, existing studies predominantly examine the pathophysiology of COPD at a single time point only, ignoring the fact that COPD is a progressive chronic disease that will develop over time. Typically in a human context, COPD is only diagnosed once a patient reaches a specific degree of morbidity, preventing a detailed analysis of how the

disease starts and progresses. Using animal models, an understanding of how the disease develops, even prior to clinical manifestations, can be obtained.

In order to develop new, preventative therapies for COPD patients, it is critical that an understanding of the initial stages of immune system activation and recruitment to the lungs and other tissues is established. As such, a model in which the progression of the disease over time, on a whole-body and multi-organ system approach and that studies the pathophysiology in both males and females represents a significant development and enhancement of existing models which typically only focus on a single tissue, sex and time point.

Data from this study will identify the key biological mechanisms involved in the development and progression of COPD in a valid mouse model. At the centre of this proposal will be a study of the immune system, its activation and its influence on multiple organ systems in the body. Findings from this study may allow for the future replacement of animal models with appropriate in vitro cell lines, whole organ and/or in silico models. In addition, following identification of key molecular and biochemical pathways involved, data from this proposal can be translated from the mouse into human samples.

What outputs do you think you will see at the end of this project?

Although COPD is a significant global health burden, the mechanisms underlying disease progression and persistence are poorly understood. Furthermore, current therapeutic interventions for COPD typically rely on smoking cessation and management of symptoms. The development of this novel murine model will provide researchers with a new model that accurately reflects human COPD in a more three-Rs friendly manner, and will maximise their data outputs in comparison to models currently in use. Although this model is designed with the investigation of immunopathogenic mechanisms of COPD in mind, the model has additional potential future applications for researchers investigating other pathological mechanisms or developing novel therapeutic interventions and treatments of COPD. The model will help to further define and identify immunopathological mechanisms of COPD, providing additional characterisation of immune cell and cytokine profiles to further solidify current understanding and research of COPD immunopathogenesis.

Throughout the duration of this project, several publications including primary research articles and review papers will be produced. Data will also be presented at relevant national and international (where possible) conferences by the principal investigators and PhD and graduate students. We are currently in the process of publishing a systematic review paper of current cigarette smoke exposure murine models of COPD that investigate immunopathological mechanisms.

The generation of a tissue bank throughout the project will provide research materials for future PhD students, graduate students and collaborators, as well as provide data for future grant applications relevant to follow on projects. Samples from this tissue bank as

well as directly from mice utilised throughout the project may also be used for additional analyses that will provide further insight into additional mechanisms of COPD or for additional investigations to elaborate on data gained throughout the project, therefore reducing the number of animals needed for further research.

Who or what will benefit from these outputs, and how?

The findings and results from this project will have many benefits to a multitude of groups within the academic and health care communities over both short (5 years) and long-term (10-15 years) time scales. First and foremost, the project will provide COPD researchers with a more refined and characterised murine model with which to conduct research that will maximise potential outputs as well as better reflect human COPD and the three Rs of animal research. As indicated above, there is a paucity of well-described and characterised models which explore the development of COPD over time, on a whole body basis and in both sexes. As such, this model will be of benefit to other research groups in this area. Upon presentation of our data at appropriate conferences and publication of our results, our model will act as a comparator against which other research groups could refine their own models to improve the welfare of their animals and the validity of their data. We anticipate the establishment, validation and publication of initial results from this novel model will occur within a comparatively short (3-5 years) time scale.

Findings from our project will aid in the further characterisation of pathological mechanisms of COPD. These findings will be of clinical relevance and may aid in the development of new approaches for the management of COPD patients. Such translational outcomes will occur on a medium- to long-term time frame (10-15 years). However, following the identification of clinically relevant mechanisms in our mouse model, we anticipate the development of a range of in vitro and in silico models. These will be based on using human samples and that will aid in the translation of our findings from the mouse to humans. These are likely to occur on a medium time frame of 5-10 years after initiation of the research.

How will you look to maximise the outputs of this work?

To maximise the output from this project, we will publish the research data in high-impact, open-access journals. Furthermore, data collected under this project will be presented at international, national and institutional conferences, meetings and seminars. In addition, we will use publicly accessible outlets such as the Conversation UK to discuss research originating from this project. Where ever possible, we will also collaborate and share resources (samples, knowledge, technical skills) with other researches (both within and outside of the University) to maximise the scope and impact of our research. It is our anticipation that all data generated under this project (both positive and negative) will be made available.

Species and numbers of animals expected to be used

- Mice: 510

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice represent the most appropriate animal model for these studies for a number of specific reasons. Firstly, patterns of COPD pathophysiology in mice in response to experimental insults (e.g. cigarette smoke and/or extract) bear significant similarity to that in humans. Furthermore, mice are the lowest vertebrate order that develop the significant lung changes associated with COPD. This means that mice represent the optimal model with which to study COPD disease progression.

Second, the un-surpassed genomic annotation of the C56BL/6 strain permits analyses into the underlying molecular and epigenetic regulation in tissues isolated from our mice, maximising the level of data retrievable from each animal. Additionally, the array of genetically manipulated mouse strains means that upon identification of potential therapeutic targets, appropriate mouse knock-out models can be investigated, providing more insight ahead of subsequent human focused studies.

Finally, the use of rodents allows for the isolation, culture, manipulation and cellular analysis of somatic and reproductive samples. As such, by using rodents it is possible to understand the entire aetiology of COPD development and progression as well as studying the impact the condition on the whole body.

Such an integrated, whole-organism approach would not be possible in humans or non-mammalian species.

Typically, what will be done to an animal used in your project?

Under this project, we will study the pathophysiological development of COPD pathophysiology in adult male and female mice in response to a cigarette smoke extract. Typically, male and female C56BL/6J will be exposed to a cigarette smoke extract created in our laboratory via an intranasal route. We have performed a systematic review of the existing literature (which is being prepared for publication) on the different animal models of COPD. Of 217 papers compiled during our literature search, only 3 studies that used a cigarette smoke extract to induce COPD in mice. None of these studies characterised disease progression over time, examined the impacts in both sexes or looked on a whole body basis.

As such, there is currently no existing data indicating what an optimal concentration or exposure duration to cigarette smoke extract is to mimic the progression of disease in humans. Furthermore, none of the studies we identified reported on the adverse effects of their models for the welfare of the animals used.

In light of the lack of detailed published literature on which to establish an optimal experimental set of concentration and dose parameters, we propose to conduct an initial pilot study. The aim of this pilot study will be to define the minimal dose necessary to observe pathophysiological changes in line with patients presenting with moderate COPD. Specifically, we will use a set of experimental endpoints typically consisting of (i) elevated lung damage (as defined by significant increases in histological assessment of alveolar mean Linear Intercept (Li) and Destructive Index (Di) parameters); (ii) statistically significant remodelling and extracellular matrix deposition of the lung vasculature; (iii)

decrease in blood oxygen saturation to 85%; (iv) significant infiltration of lung immune cell populations into the lung tissue and; (v) elevated peripheral immune cell activation and inflammatory mediator profiles.

To ensure our pilot study has the minimal impact on animal welfare, we will adopt a system in which mice (males and females) are initially exposed to the lowest concentration of extract (typically 1%) and for the shortest duration (typically 1 week). Animals will be exposed to the cigarette smoke extract three time a week via an intranasal route and will be compared again control (culture medium only) animals. Once a week, mice will be restrained for the conscious measurement of blood oxygen saturation and heart rate using a pulse oximeter. At the same time, we will collect a tail vein blood sample for the measurement of circulating inflammatory markers and immune cells using a multiplex protein microarray and spectral flow cytometry within our laboratory. Our new spectral flow cytometer will allow us to characterise and identify the differentiation of key immune cells involved in COPD pathogenesis (NK/NKT/CD8+ T cells, Macrophages and Th17/Tregs) using up to 40 key markers in a single sample. We are currently validating this novel flow panel in vitro, and will apply it to blood samples obtained from the in vivo murine model. In addition, multiplex protein microarrays will allow us to analyse and quantify the presence of inflammatory modulators such as cytokines within the blood samples. Both of these techniques will allow us to characterise immunopathogenic mechanisms beyond what has already been previously identified in literature and within our own in vitro studies, and require a minimal amount of samples to obtain a significant amount of data.

At the end of the defined exposure time, mice will be culled for the collection and study of a range of tissues. Typically, after humane killing by a schedule one technique, lungs will be flushed using sterile phosphate buffered saline for the collection of bronchoalveolar lavage fluid (BALF) and analysis of immune cell populations. Lung tissue will then be fixed and processed for histological assessment of vascular smooth muscle remodelling and alveolar damage. Other tissues such as the liver, kidney, heart and reproductive tissues will also be collected, processed for histological assessment. Peripheral blood will be collected for analysis of immune cell and inflammatory mediator profiles as described above. If no change in the experimental and physiological endpoints described above are noted, in addition to no detrimental effects on animal welfare (see comments below), the dosing regimen will be increased incrementally (typically by an extra 2% concentration of the extract and for an extra 2 weeks of exposure). Based on existing evidence that onset and severity of COPD displays sex-specific differences, it is conceivable that to establish

the same COPD pathophysiological status, the dosing regimen between males and females may be different.

During this Pilot Protocol we will monitor closely the welfare of the animals (with supervision from the NVS and NACWO, or appropriate deputies) for signs of weight loss, changes in body condition and breathing difficulties. Under this pilot study our humane endpoints will be a loss of body weight of ≥20%, a body condition score of 2 or any animal found to be making significant respiratory effort with no improvement in symptoms over the course of 4 hours. In addition, animals will be scored for clinical symptoms on a 0-3 scale (where applicable) and a cumulative score of 12 will result in the animal being humanely killed. The findings from this pilot will inform on the dosing regimen required to induce a phenotype reflective of moderate COPD in humans, and to ensure our experiments are maintained within a ‘Moderate’ severity limit or lower.

Under Protocol 2, male and female mice will be exposed to the defined, optimal dosing regimen, as established under Protocol 1. Here, the aim of this Protocol is to define the biological mechanisms driving the progression of COPD and to understand the potential recovery of the mice once administration of the extract has been stopped. As under the Pilot protocol, mice will be exposed to the cigarette smoke extract three times a week. Mice will be subjected to regular (weekly), non-invasive monitoring of blood oxygen saturation and heart rate via pulse oximeter as well as tail vein blood sampling. At the end of the dosing regimen, one group of the animals will be culled for the collection of tissues for molecular analysis and in vitro characterisation of isolated cells e.g. vascular smooth muscle cells.

As sex differences in respiratory physiology and predilection for developing COPD have been documented, there may be connection between sex hormones, reproductive health indicators and disease pathogenesis. A separate group of experimental males and female will be mated to control animals for the analysis of reproductive health and correlated to extent of disease progression. Here pregnant females will be culled at distinct stages of gestation for the collection of reproductive, embryonic and fetal tissues. All pregnant females will be culled prior to term.

Finally, to address whether the induced tissue damage and activated immune cell profiles can return to a more normal profile once the exposure to the cigarette smoke extract stops, a second group of animals will be kept and monitored at either 1, 5 or 10 weeks after their last exposure to the cigarette smoke extract. Analysis of these animals will be in line with the experiments detailed above and will inform on how their physiology responds after cessation of exposure to the cigarette smoke extract.

What are the expected impacts and/or adverse effects for the animals during your project?

The intention of this study is to induce pathophysiology symptoms in line with moderate COPD in humans. Our Pilot protocol has been designed to ensure the welfare impact on the animals is minimised and remains within a ‘moderate severity’ limit.

During this program of work, we will monitor closely the welfare of all animals (with supervision from the NVS and NACWO, or appropriate deputies) for signs of weight loss, changes in body condition and breathing difficulties such as an increased respiration rate and breathlessness. Under this pilot study our humane endpoints will be a loss of body weight of ≥20%, a body condition score of 2, any animal found to be making significant respiratory effort with no improvement in symptoms over the course of 4 hours or a drop in oxygen saturation below 85%. In addition, animals will be scored for clinical symptoms on a 0-3 scale (where applicable) and a cumulative score of 12 will result in the animal being humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

As indicated elsewhere, there is a significant paucity of published data on the welfare impacts of administering a cigarette smoke extract to mice. As such, it is difficult to anticipate precisely the likely severities that animals on this application will experience. However, to mitigate this lack of prior knowledge, the humane endpoints associated with our protocols have been set to ensure that all experiments will not exceed a 'moderate' severity limit.

Our pilot study has been designed such that animals will be exposed initially to the lowest concentration of the extract and for the shortest time. Dependent on the experimental endpoints observed, in conjunction with our humane endpoints, the dosing regimen may then be increased if minimal changes are observed. As such, it is possible that our experimental endpoints are achieved with minimal impact on animal welfare. However, it is also possible that the anticipated changes in lung and vascular histology, oxygen saturation and inflammatory status may impact to a moderate severity in those animals receiving the higher doses and for long durations. It is anticipated that under Protocol 1, 25% of animals may experience Moderate severity effects in response to the higher doses of cigarette smoke extract. If it is the case that we establish a regimen that achieves our experimental endpoints but which are associated with Moderate severity effects, then all animals under protocol 2 will experience Moderate severity effects. These are anticipated to be weight loss (no more than 20%), decline in body condition (no lower than 2), respiratory impairment (laboured breathing and/or increased respiration rate lasting no longer than 4 hours) and/or a decline in blood oxygen saturation (no lower than 85%).

Separate to the impact of the cigarette smoke extract on animal welfare, we anticipate mild severity short-term effects on animals from regular restraint, sampling of blood from the tail

vein and monitoring of blood oxygen saturation and heart rate. These effects will typically be in the form of soreness and swelling at the site of needle insertion, or short-term stress from the restraint. These are likely to occur in all animals but are not anticipated to have lasting harm.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The purpose of the work is to determine the effects of a cigarette smoke extract on the development of COPD and its progression. Such studies, while of significant benefit to human health, would be considered un-ethical in humans. Therefore, to achieve the aims of this project, the use of animals is necessary to cover the complex, inter-related, whole body nature of the study.

Which non-animal alternatives did you consider for use in this project?

As COPD is a systemic disorder, appropriate models are needed which permit the analysis on a whole body basis. Therefore, in vitro or isolated organ systems do not provide sufficient interrogation with which to determine whole body effects of cigarette smoke exposure on COPD development.

Why were they not suitable?

The central aim of this project is to examine the effects of a cigarette smoke extract on whole body physiology and disease progression in a clinically relevant model. While complex in vitro systems have been developed to study central biological processes e.g organ on a chip, organoids, 3D-scaffolds for cell culture, these models represent isolated systems in vitro. Therefore, in order to understand how a cigarette smoke extract affects lung function and physiology, inflammatory status and peripheral organ structures (all interconnected) in an undisturbed system, live animal research is necessary.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The appropriate number of mice to be used has been determined through the use of the NC3R's online Experimental Design Assistant and existing published literature of mouse models of cigarette smoke extract. Pathophysiological data on lung morphology, circulating inflammatory markers and immune cell populations (all parameters that will be assessed under this Licence) have been taken from relevant publications (He et al, 2015, Tob Induc Dis. 13(1):6; Tabata et al. 2015. Int Immunopharmacol. 25(2):511-7; Braber et al. 2011. Am J Respir Crit Care Med. 185(8):817-24).

Using these resources, we have calculated that an n of 10 animals per group would be required to observe significant differences within any single treatment or control group. As the majority of existing studies only assess the pathophysiological impacts of COPD in a single sex, and sex-specific differences in the responses to the cigarette smoke extract are anticipated, an n of 20 animals (10 males and 10 females) per group (either treatment or control) would be assessed for each individual experiment.

Based on these factors, it is anticipated that under Protocol 1, a maximum of 200 mice will be required, while under Protocol 2, a maximum of 310 mice will be required

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical advice was sought to devise an analytical approach which allowing us to take into account the multiple levels of interaction within our data and other potential confounding variables (e.g. sex, body weight, age, dosing regimen). Using this approach we are able to factor into our statistical analyses the multiple measurements from individual animals and any potential extract 'batch' effects. Such analyses allow us to maximise the information gained from any one individual animal and so reduce the number of mice required.

In addition, we have used the NC3Rs experimental Design Assistant to confirm that an n of 10 males and females per treatment group are necessary to observe significant changes in lung function and morphology, circulating inflammatory mediators and altered immune cell populations based on existing published data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, multiple measurements will be taken from any one individual animal prior to culling for the collection of tissues for subsequent molecular and biochemical analyses. This will ensure maximal data is obtained from mouse and will minimise unnecessary repetition of experiments.

From all animals used within this project, a range of tissues will be collected and stored for later analysis. These will be used for under-and post-graduate studies within our

laboratory, for the generation of preliminary data for further grants and will also be made available to collaborators where appropriate.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The aim of this project is to use a cigarette smoke extract for the induction and study of COPD progression in mice. Many of the hallmark features of human COPD, namely (i) lung inflammation, (ii) impaired lung function; (iii) emphysema; (iv) mucus hypersecretion and (v) airway and vascular remodelling, can be induced in response to cigarette smoke. However, the animal welfare issues and modest scientific benefits associated with rodent chronic cigarette smoke aerosol exposure means alternative models are needed. Exposure of mice to a cigarette smoke extract (via an intra nasal route) have been shown to result in the same pathophysiological changes as those observed in restrained animals exposed chronically to cigarette smoke aerosol. Therefore, the use of such extracts represents a significant refinement (reduced handling, minimal number of exposures) over older, whole body- exposure methods which required long-durations of restraint.

Mice represent the most appropriate model for use in these studies as they represent the lowest vertebrate species that develops lung injury, lung inflammation and pulmonary fibrosis in response to various challenges. As such, the use of non-mammalian species, such as insects or fish, would be inappropriate for the proposed series of studies. Additionally, aspects of mouse physiology, central to understanding the whole body disease progression, share significant similarity with humans.

Mice provide a further experimental benefit through their unparalleled genomic annotation. After cull, numerous tissues will be collected and stored for down-stream detailed investigations into the genomic and epigenomic mechanisms underlying the development of COPD. The detailed annotation of the C56BL/6 genome, transcriptome, proteome and epigenome will allow for maximal levels of data to be retried from each animal. For these reasons, rodents provide the most appropriate non-human model for such studies.

Why can’t you use animals that are less sentient?

COPD is a complex whole body condition that develops over time and typically within adults. In order to better define and understand the development and aetiology of this disease, complex whole body models are needed. As mice are the lowest mammalian

species to develop the pathohysiological symptoms of COPD, then the use of less sentient animals would be of lower disease relevance to humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals studied under this protocol will be monitored weekly for weight gain/loss, general condition, demeanour and behaviour. Any significant deviations from normal will be recorded and, where appropriate, result in discussions with staff withing the facility, the NVS or their deputy and appropriate action enacted.

In all protocol steps where dosing/substance administration is required, maximum volumes will not exceed LASA guidelines.

Animals will be humanely killed via a scheduled one method if they show other than transient signs of ill health such as pronounced piloerection and hunched posture (score of 3/3 on health monitoring record), inactivity, or inappetance for a period greater than 24 hours. Any animal with a cumulative health score of 12 or above will also be humanely killed. Any animal that loses 20% of its body weight, records a body condition score of 2 or shows serious clinical signs such as diarrhoea will also be killed. Any animal making significant respiratory effort such as breathlessness or increased respiration rate (respiratory score of 3/3) and/or laboured breathing (laboured breathing score of 2/3) for a period of up to 4 hours with no improvement in symptoms will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Throughout the course of this project we will make reference to multiple sources for best practice guidance. these will include:-

Fund for Replacement of Animals in Medical Experiments: www.frame.org.uk

NC3R’s website for details on experimental design: https://nc3rs.org.uk/experimental-design NC3R's ARRIVE guidelines: https://www.nc3rs.org.uk/arrive-guidelines

Laboratory Animal Science Association (LASA) publications: https://www.lasa.co.uk/current_publications/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I currently subscribe to the NC3Rs monthly e mail alerts which keep me updated to the most recent developments in NC3Rs publications, guidance and information.

We also receive regular updates and latest animal welfare guidance information via bulletins disseminated from our Biomedical Research.

Finally, information is disseminated and methods of best practice are discussed via our establishment users meetings of which my research group are involved with.

58. Improving our understanding of the bovine immune system

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Immunology, Bovine immune response, Pathogenesis of disease, Mastitis

Animal types	Life stages
Cattle	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to take a multifaceted approach to improve our understanding of the bovine immune system (the tool kit to fight infection), both in the 'healthy' animal and when faced with 'insults', such as infection with a microorganism. It will consider the development of the immune system, from a neonatal calf through to a lactating (in milk) cow; from the start of an animal's life through to adulthood.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit – these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The current Dairy Cattle Welfare Strategy lists priority areas, including lameness, calves/youngstock, promoting higher welfare, mastitis, fertility and breeding, as issues that are challenging the dairy industry. With global demand for dairy products expected to grow by ~2%/annum over the next 10 years and a potential for UK growth post-Brexit, it is key that research gaps around these major challenges are addressed.

This research will advance our understanding of basic bioscience underpinning health, the stages of development of key diseases, including mastitis, and how immune system development impacts on healthy ageing, potentially maximising the health, welfare and productivity of the dairy cow and the sustainability of dairy farming, benefitting animals, dairy farmers and consumers. The UK has ~1.8 million dairy cows that could benefit.

This research will identify key roles of the immune system across the ages, from early life 'insults' into their productive adult life, and a deeper understanding of the key issues that impact the industry and cattle health welfare. It will advance the science of both the fundamental biology of the immune system as well as provide potential targets for intervention or vaccine candidates.

What outputs do you think you will see at the end of this project?

This research will advance our understanding of basic bioscience underpinning health, the progression/development or pathogenesis of key diseases and how immune system development impacts on healthy ageing, potentially maximising the health, welfare and productivity of the dairy cow and the sustainability of dairy farming, benefitting animals, dairy farmers and consumers. The UK has ~1.8 million dairy cows that could benefit.

Intended outputs

This research will identify key roles of the immune system across the ages, from responding to early life 'insults' into their productive life, and a deeper understanding of the key issues that impact the industry and cattle health/welfare. It will advance the science of both the fundamental biology of the immune system as well as provide potential targets for intervention or vaccine candidates. The research will provide a wide array of data including whole genome sequencing, correlations between an animal's genetic make-up (genotype) and an observable trait (phenotype) and immunological/gene expression data. All of these will results in publications in peer-reviewed journals, as well as dissemination at conferences and stakeholder events.

Who or what will benefit from these outputs, and how?

Who?

The outputs will be used by researchers investigating the bovine immune system, bovine diseases and others interested in the age-specific response. The research could directly inform the optimal management of dairy calves in order to minimise exposure to early life challenges (these can be infections or stressors) and may inform age-specific vaccine

design. Results will be presented directly to AHDB Dairy (levy board) for use in knowledge exchange activities, farmers and the research community/policy makers via peer reviewed publications and conference presentations.

How?

The outputs would provide a scientific understanding of the differences in the immune system through the key life stages of the dairy cow, a current research gap, which could be harnessed for therapeutic/vaccination regimes as well as management strategies. A greater understanding of disease pathogenesis will provide avenues to investigate for diagnostics, vaccine/vaccine adjuvant candidates and in breeding for greater resistance to the development of a disease.

Short-term benefits

Researchers will benefit from a better understanding of the immune response, particularly through the key life stages. Findings will be made available to scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings.

Pharmaceutical industries/farmers may benefit through improved vaccination regimes, particularly with regards to neonates. In addition, this research will lead to investigations into therapeutic targets, vaccine adjuvants and candidate vaccines. Identifying correlates of disease (essentially the fingerprint of a disease and how that changes as a disease progress) may lead to the development of novel diagnostic tests, enabling the potential for an infection to progress to disease much earlier. We will work with farm vets/farmers on the impact of early life insults on the long term health of the dairy cow. There is the potential to improve cow welfare (and therefore enhanced consumer confidence) and improved sustainability through improved long-term cow health and productivity.

Medium and long-term benefits

In terms of sustainability, benefits of the research would include a reduction in greenhouse gas emissions, carbon and non-renewable energy consumption, through improved health and reproductive performance during the life of a dairy cow, with potential to improve their life span. In terms of financial return to farmers, small improvements in calf survival, health and fertility would lead to large increases in efficiency and profitability. The potential financial benefits extend beyond those associated with health and production, as the image of dairy farming is critical to consumer confidence and the long- term sustainability of the industry.

How will you look to maximise the outputs of this work?

All work covered by this application will be funded by funding bodies who will expect all new knowledge to be disseminated as detailed in the Impact part of any grant application; whilst UKRI have recently removed the standalone Pathways to Impact document this will

still form a key part of any funding application. Dissemination will be through publications, conferences and workshops ensuring to involve all stakeholders, with data made freely available on appropriate platforms. The in vivo studies, funded through a BBSRC grant, will all be using well established protocols for the infection studies, therefore we do not anticipate any unsuccessful approaches. The remainder of the work is primarily of a fundamental nature, providing new knowledge and again we would not expect any unsuccessful approaches. Should this occur we would ensure that that approach is part of a bigger publication.

Species and numbers of animals expected to be used

- Cattle: 190

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In humans, several differences between lymphocyte (white blood cell) subpopulations obtained by immunophenotyping (determining the immune cell profile) in healthy neonates, infants and adults have been reported. In neonates and infants, lower levels of lymphocyte function, compared to adults have been described. Healthy neonates/infants react differently to antigens and are less efficient in coping with some microorganisms. Their lymphocytes undergo maturational changes that are essential for normal development.

Previous studies in cattle are now out dated, with the discovery of novel cells and immune markers (parts of the cell that enable them to be identified, such as T-helper/Th17 cells and interleukin (IL) -17 protein family, the need for an updated and thorough comparison across the key life stages is essential. This combined with an understanding of their comparative response to key microorganisms has the potential to improve animal health and productivity.

With UK farmers experiencing continual losses of potential replacement adult cows throughout their rearing period with ~8% calf mortality, 14% of calves failing to reach first calving, and a further 15% of cows culled in their first lactation (milking period), a better understanding of how early life insults impact on animal health aims to reduce these losses. The project will generate evidence to support the age-specific immune phenotype of the dairy cow from birth through to adult life, demonstrating how key immune cells respond to stimulation with key microorganisms.

Specifically the project will address two key areas:

1. How does the type, proportion and functionality of key immune cells differ with age and how do they respond to key microorganisms?

2. Greater understanding of disease pathogenesis, particularly with regards to mastitis.

Typically, what will be done to an animal used in your project?

The project involves simple protocols unlikely to cause any harm or distress to an animal, these include the use of a blunt cannula (very similar to a syringe without the needle) to remove milk aseptically from the udder and the use of a swab to sample faeces. Both of these protocols would involve the reuse of animals to allow the monitoring of milk immune cells and bacterial carriage (present but causing no adverse effects) over time.

Obtaining blood by venipuncture (removal from a vein) is unlikely to cause more than transient discomfort. Animals may however be re-sampled (continuous use) for analyses studying immune responses through the life stages, and the risk of haematoma formation or infection will be minimised.

Infection studies involve the experimental infection, through the use of a blunt canula directly into one/two quarters, of lactating heifers (first milking) in order to investigate the pathogenesis of mastitis. This may involve taking blood during twice daily milking. Infection studies will last no more than four days (excluding acclimatisation and monitoring pre- and post-study respectively), with animals treated, monitored prior to release from the study; typically two weeks in complete duration.

What are the expected impacts and/or adverse effects for the animals during your project?

The protocol with the highest severity (moderate) is the infection studies, with ~10% of animals expected to be part of studies where this level of severity may be expected. Pain, from the onset of clinical mastitis, is likely to coincide with the study end point, with animals then receiving antibiotic treatment, in addition to pain relief where necessary. Typically animals will be very responsive to treatment, resulting in a very short period (one/two days) before they start to improve.

The majority of animals to be used in these studies will see the animals released back into the herd.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The infection studies uses adult cows only, and is the only protocol with a moderate severity. Of those animals used for infection studies, <15% are likely to experience moderate severity.

All other protocols have a mild severity rating.

Collection of milk aseptically uses adult lactating animals, and when performed competently is a painless procedure that is very similar to the administration of intramammary antibiotics; as such <5% of animals will experience mild severity.

The remaining two protocols (both mild) will use animals across all life stages.

What will happen to animals at the end of this project?

Killed
Kept alive
Used in other projects
Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This work aims to investigate the

(i) Immune phenotype of the dairy cow from birth through to adult life

(ii) Age-specific response of key immune cells from dairy calves/cows at key life stages

(iii) Health and physiological responses of lactating dairy cows kept under experimental conditions and infected with microorganisms that cause mastitis.

Immune phenotyping and the comparative immune response of immune cells must be done from the target species. Dairy cows are the subject of, and direct beneficiaries of the research; as such it is not possible to conduct this work without using animals and no other alternatives are suitable. The project aims to further investigate the bovine immune response providing data that will benefit the health and welfare of this key livestock species.

In vitro studies (performed in a lab with no animals) will integrate with the data from in vivo studies; looking at both the individual role of immune cells in the milk and the pathogenesis of the disease in vivo to get an understanding of how the cells interact and the cross talk between them. The work will investigate the healthy immune system and how it correlates with other body systems and immune responses of dairy cows in response to infection in order to optimise the health and welfare of farmed dairy cattle.

Which non-animal alternatives did you consider for use in this project?

None are suitable

Why were they not suitable?

The immune system of a cow is multifactorial in nature, involving communication between various cell types plus the recruitment of other cells to the site of infection, e.g. neutrophils (a specific white blood cell) crossing from the blood stream into the mammary gland/udder. Understanding the role of key cells and their part in the immune response is essential if we are to improve the health of the dairy cow; this cannot be recreated in a non-animal alternative.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each protocol has a proposed number of animals associated with this. Protocol 1 - 60 animals

Protocol 2 - 20 animals

Protocol 3 - 50 animals

Protocol 4 - 60 animals

This estimate assumes that some of the animals used for one protocol (e.g. blood sampling 1 and faecal collection 4) may or will be used in another (e.g. milk sampling 2) as part of the same experiment; this is particularly true of animals that form part of the immune system through the life stages. Equally if correlating microbial carriage (in faeces 4) with response to different microorganisms (through the stimulation of blood cells, 1).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the NCRs Experimental Design Assistant (https://www.nc3rs.org.uk/experimental-design- assistant-eda), thereby improving transparency of our experimental design, and allowing us to share/discuss plans with colleagues and collaborators. This will provide support for randomisation, blinding and sample size calculation, and ensure that confounding variables, such as age and days in milk, are accounted for, and that each experiment will yield robust and reproducible data, ensuring that the data from every animal is utilised to its full potential. Thereby reducing the number of animals required and maximising the information obtained.

In addition:

Protocols 1 and 2: Within animal controls can be used for in vitro cell assays and stimulations, such that a proportion of each cell type from each animal being analysed is always used a control; for example a media-alone control if comparing their response to a particular microorganism grown in that media.

Protocol 3: Once data from a representative control group has been determined for a series of infection studies following strict protocols, this will eliminate the need for additional control groups, thereby reducing animal use. Animals will be randomly assigned to groups where possible/relevant.

Protocol 4: This protocol is around analysing the carriage of microorganisms that cause mastitis with the GI tract, with sampling numbers determined by the exact question being addressed alongside experimental design.

Data will be analysed using appropriate multilevel statistical techniques. For every experiment, we will write an experimental protocol which will include a statement of objective(s), a description of the experiment, covering such matters as experimental procedures, size of experiment (number of groups, number of animals/group, power calculations), how animals were allocated, and experimental material and an outline of the method of analysis of results, statistical tests, significance level and confidence limits plus treatment differences to be estimated.

I confirm that the experiments will be conducted and reported in a manner that will allow publication according to the ARRIVE guidelines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The project will ensure that the number of animals used is both minimal and appropriate. Confounding variables will be minimised, for example the use of age-matched, parity (how many times an animal has been in milk)-matched, stage of lactation (lactation is on average 305 days) matched animals where appropriate.

For work requiring blood or milk, any optimisation of laboratory work or pilot studies can utilise cells obtained through a non-ASPA method; e.g. direct from the abattoir and bulk tank milk/milk from the robot sampler.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Dairy cows are the subject of, and direct beneficiaries of the research proposed herein. The effect that a deeper understanding of age-specific immune responses, pathogenesis of key diseases including mastitis and immune phenotypes of dairy cows across the key life stages cannot be assessed in another species. Holstein Friesian cows have been chosen as this breed makes up the majority of the UK dairy herd, meaning the results of this study will be applicable to most animals in the UK.

Throughout the experimental work, many of the animals will not be removed from their normal living environment, with no change to management or social grouping (those used for protocols 1, 2 and 4). Those that are used for infection studies (protocol 3) will be taken from the same social grouping where possible to minimise stress on moving to the containment area - Barn 2, and will undergo a period of acclimatisation to the new environment before the work commences; investigating mastitis in the target species is essential for a further understanding of the pathogenesis of disease.

The experimental work in cattle will involve the predominant microorganism that causes mastitis here in the UK, namely Streptococcus uberis, and this work will use a long established experimental model of infection where the infectious dose using a virulent strain results in reliably repeatable outcomes, including detectable increases in the numbers of cells (cell counts) in the milk by 24hr post-infection. This ‘tried and tested’ approach represents the most refined model to obtain the required scientific data needed to meet the objective. As such, our choice of animals (the breed representative of the UK dairy herd), the use of first time mums/heifers (ensuring no previous cases of disease) and approach (using a well-established model) is the most refined for the intended purpose.

The methods have been refined using the following approaches:

1. To limit disturbance to the animal’s normal regime, many of the animals will not be moved from their normal environment, with blood sampling when animals are temporarily restrained for bleeding, faecal collection or sterile milk collection. Pain relief is not considered necessary for these procedures to be undertaken as it is not required for routine blood sampling, a rectal examination or the removal of milk or infection via a cannula, which is comparable to the application of intramammary antibiotics.

2. The duration of experiments and the number of procedural steps will be limited to the minimum necessary to collect robust data. Bleeding will be no more than twice a week except in the infection studies where blood may be taken at milking, with the smallest gauge needle used to minimise potential for the development of a haematoma (bruising).

3. Where ever possible individual procedural steps will be combined (e.g. blood sampling and infection), to minimise extra stress through handling.We consider three of the four procedures in our protocols to be of mild severity. Bleeding is considered to cause only

mild transient discomfort to the cattle. To minimise any discomfort all staff will be trained and experienced in handling and blood sampling cattle, and the smallest size needle appropriate will be used. Insertion of a cannula to remove milk, when performed by trained and experienced staff, should cause no discomfort as it is directly comparable to the application of intramammary (into the mammary gland) antibiotics. Collection of faecal samples should also cause no discomfort as it is comparable, but far less intrusive, than a rectal examination.

The third protocol, involving the experimental infection or one/two mammary quarters per cow is considered to be moderate in severity because for some of the work will be treating as the signs of clinical mastitis become evident. The model of infection used is very well established, and the timing and nature of clinical signs well documented. Together with the monitoring of somatic cell count (cells in the milk) at each milking (results will be generated almost immediately), we will end experiments and begin treatment based on a wide array of information; more than that available in a typical dairy environment. We may see some hardening and inflammation of the infected part(s) of the udder, but these will be humane end points. Animals will be monitored through scoring of the four quarters that make up the udder, their milk and animal demeanour/behaviour.

In addition, current funding is around the early stages post-infection, therefore these animals will be treated with few if any signs of clinical mastitis.

Why can't you use animals that are less sentient?

Our choice of animals is the breed representative of the UK dairy herd. Some of the proposed work will be looking at the development of their immune system through the life stages with the overall aim of improving their health and welfare through a better understanding of their immune system, pathogenesis of diseases which are relevant from an animal health and welfare perspective. This has the potential to ensure each cow is as healthy as she can be, extending her productive life and therefore reducing the need for increases in cow numbers when increases in productivity can meet demand.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

While three of the four protocols are considered 'mild', each animal will be monitored after a procedure to ensure they are not suffering any adverse effects. To minimise any stress from handling, food 'rewards' may be offered during, for example when using a head yoke to facilitate faecal sampling, or after a procedure, for example following venepuncture. The latter would also allow for monitoring of the site to ensure blood flow had stopped.

For those animals used in protocol 3 (infection studies), allowing for the animals to get used to their new surroundings prior to the study starting is essential to minimise their stress. Typically the animals will be monitored twice a day at time of milking, but as a complete infection (through to humane end point) study end point nears a third monitoring time will be added. Most of the studies planned are around the early stages of disease

and, as such, studies will be ended before this extra monitoring is needed and before many if not all signs of clinical mastitis are observed.

Post-procedure, analgesia/pain relief (in addition to local intramammary therapy used at the study end point plus systemic antibiotics were needed) will be administered if the animal is thought to be showing any signs of pain.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

3Rs resources available at https://www.nc3rs.org.uk/3rs-resources.

Animal Research: Reporting of In Vivo Experiments guidelines available at https://www.nc3rs.org.uk/arrive-guidelines.

The general principles for blood sampling are available, including the safe volumes for a single bleed for cattle - this resource will be regularly reviewed for updates to the guidance.

https://www.nc3rs.org.uk/blood-sampling-general-principles

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I subscribe to monthly updates from the NC3Rs on funding opportunities, events and publications, and have utilised and will continue to utilise the many resources made available by them. Before work commences I will ensure that new advances or recommendations are incorporated into the planning thereby ensuring their timely and effective implementation.

59. Defining osteoarthritis mechanisms

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

osteoarthritis, musculoskeletal health, trauma, ageing

Animal types	Life stages
Mice	embryo, adult, juvenile, neonate, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to define novel mechanisms implicated in osteoarthritis (OA) development, as potential targets for therapy.

This will include answering the following questions:

1. How does abnormal extracellular matrix components, such as Fibrillin-1, contribute to OA development?

2. How does growth factor signalling modulate OA progression? (how are growth factors expression regulated in musculoskeletal tissues during development, growth and ageing, and with OA development? How does the extracellular matrix further regulates growth factor activity? (growth factors include TGFβ, CCN family members, BMPs)

3. Which cell subtype contribute to joint health and OA development?

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Osteoarthritis (OA) is the commonest chronic disease, affecting almost 9 million people in the UK. The World Health Organisation estimates that 9.6% of men and 18.0% of women aged over 60 already have symptomatic OA, making this disease one of the ten most disabling disease in humans. OA affects joints and include pathological changes and degeneration of articular cartilage, synovial membrane hyperplasia and fibrosis, sclerosis of subchondral bone, osteophyte formation, meniscal and ligament pathologies. These all contribute to pain and restriction in range of movements of the joints. Despite the prevalence of OA, there is currently no disease modifying treatment to slow the progress of the disease. As the aged population is increasing, new treatments for OA will only become more needed.

What outputs do you think you will see at the end of this project?

Outputs will include the generation of new understanding of how the musculoskeletal system forms and functions to allow for healthy joint ageing, and to define mechanisms by which osteoarthritis develops. This new understanding will lead to potential new therapeutic targets to prevent or reduce OA development.

All of these data will also be published in open access journals to ensure these new information are shared equally to other scientists, clinicians and the public.

Who or what will benefit from these outputs, and how?

The main beneficiaries of this applications are the human and animals that develop OA, either due to genetic predisposition to joint degeneration, or following ageing, joint injury or altered mechanical environment.

They will benefit from advances in our knowledge of how to combat this disease and will be the beneficiaries from drugs that are developed to target molecules that we have shown to be critical to the development of the disease.

The other beneficiaries are scientists around the world who are working on this and related diseases, including pharma companies developing drugs that can manipulate some of the molecules that are highlighted in this programme of work

How will you look to maximise the outputs of this work?

We will disseminate our results periodically through presentation and discussion at national and international conferences, as well as by open access publication. In addition, collaborations across the world with expertise in complementary areas of research, or with

specific skills, methods or material of interest will be sought out to complement our work. Conversely, our data and biological material created through this project (such as new transgenic mouse lines) will be shared openly.

Species and numbers of animals expected to be used

- Mice: 9,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The animals used in this project are mainly transgenic mice. Mice are genetically tractable mammalian system. We will be using all stages of a mouse lifecourse to be able to answer our questions relating to joint health and osteoarthritis development (a chronic age-related disease).

Throughout the different aims of our project, we will:

1. Define how our cell or molecular target of interest contributes to normal limb development and growth. These events are shown to contribute to OA susceptibility and development.

2. Understand the mechanisms involved in ageing- and trauma-induced OA. For these adult and aged mice will be used.

3. Develop new transgenic lines in order to assess the origin and function of cell subtypes in musculoskeletal tissues across the lifecourse and in response to joint trauma. Development of new transgenic lines involves genetic manipulation in early embryogenesis (either via CRISPR-Cas9 systems or via transgene expression).

Typically, what will be done to an animal used in your project?

Various manipulations will be used depending on the specific question and experimental set up. A few examples as described as follow:

- Creation of new transgenic line. Targeted deletion of a specific genetic segment will first be identified in silico and tested in vitro. New mouse lines for targeted gene manipulation will be created using one-cell stage embryos microinjection of the required components and their transplantation into pseudo-pregnant female mice. A similar method is also used for expression of a specific transgene (for example to follow a cell that may be expressing a specific gene). Pups born will be genotyped and selected as founders for new transgenic lines to be bred. Following appropriate breeding regimes, homozygote KO (both alleles

deleted) lines will generally be used, with a separate WT line (to reduce waste of Heterozygote animals that will not be used).

- Induction of post-traumatic OA (PTOA). Adult mice (usually from 10wks of age) are used. The right knee and ankle are mechanically loaded at specific magnitudes (40 cycles of 9N is sufficient to induce PTOA without ligament rupture) 3 times per week for 2 weeks, under Isoflurane anaesthesia. After a specific period of time, mice are killed via an accepted schedule 1 method and tissues processed for analysis. Non-invasive gait monitoring may be used throughout the experiment, starting 1 week before the first loading episode, followed by weekly measures for a month, and bi-monthly after that. Treatment with either Tamoxifen (for induction of the Cre-recombinase activity in specific transgenic mice) or with a drug of interest will be used when appropriate. Some mice may also be kept in specific cages allowing for voluntary exercise: mice will be individually housed in cages containing wheels; full information including time used on the wheels and distance run are automatically recorded in this system. Control animals in identical cages, but without the wheels, will also be used in parallel.

- Ageing-induced OA. Mice will be left to age up to 72weeks of age. During this time, gait may be assessed on a monthly basis. As above, treatment and voluntary exercise set up may be used.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the transgenic mice that we use and will be generating fall under mild category, because we express a transgene that does not cause harm (for cell tracing). The transgenics in which we will be deleting a gene, such as Fibrillin-1, do show spontaneous limb weakness and increased OA development. These are therefore classified as “moderate". In addition, mouse ageing to 72wks and induction of post-traumatic OA will also be classified as “moderate” due to the development of joint disease. For the joint loading model (used for induction of post-traumatic OA), some possible redness and swelling of their loaded limb is possible in a small proportion of mice, but are generally only seen during the loading period.

These models have now been used extensively and we know they do not affect the ability of the mice to freely move in their cages and access food or water.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild: majority of mice being bred.

Moderate: all mice following post-traumatic OA induction; a majority of mice aged over 52wks of age.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to define mechanisms that control joint degeneration in osteoarthritis, which is a slow degenerative disease, that affects various tissues within a joint and is often dependent on the physiological setting of the individual (such as diet, genetic background, lifestyle, etc).

here are 3 main reasons why this cannot be achieved without the use of animals:

- The time needed for the disease and the mechanisms to take place, mean that it is not possible to achieve in vitro (in humans, disease can take place over decades)

- The joint is a 3D system, with important interactions between cells and extracellular matrix, with strong cross talk between tissues that comprise this organ, all of which are highly mechanosensitive. There are currently no in vitro systems that can recapitulate all of these aspects together.

- It is becoming also clearer that other organs may influence disease progression (e.g. immunity, metabolic events, gut flora).

Which non-animal alternatives did you consider for use in this project?

When possible and relevant to the research question, we will use already published datasets to investigate initial results: this may include publicly available multi-OMICS datasets, and in silico models used for promoter-enhancer activities. In addition, when human samples are available, these will be used before investigating the mouse model. This ensures the specific mechanism is a likely target, that can then be confirmed in mouse models.

Before generating any new transgenic mice, we will be searching KOMP and IMPC and any cryo banks of frozen embryos in order not to duplicate the creation of mouse transgenic lines. In addition, we are freezing down our own lines and making them available to the community at large.

Why were they not suitable?

It is well accepted now that multiple cells and organs are involved in OA and studying each separately is not sufficient to replicate the in vivo situation. In addition, each tissue in the joint is rich in a complex extracellular matrix that we have yet to be able to replicate in vitro.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in these studies is calculated from power analysis based on similar previous studies. Indeed, taking into account the standard deviations in previous studies and the primary outcome expected in order to reach clinical significance, it has been estimated that n=15 animals per group must be used in OA models.

In addition, we account for breeding issues and the rate of mice of the right genotype being born.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Careful design of the experiments must be implemented to avoid unnecessary use of animals. The use of software such as the NC3Rs Experimental Assistant Design will ensure adequate planning before the start of experiments (including randomisation and blinding when possible).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Appropriate controls are also used: all studies use aged and gender matched WT controls; control for treatment such as loading include sham mice. The contralateral leg is not used as a control as we have shown significant changes in this limb.

Re-use of ex-breeders to the ageing protocol also serves to reduce the number of animals used. These have already been kept for 6 months.

We have extensive experience in the use of mouse models to study our questions, with appropriate skills necessary for the best tissue preparation and analysis. When a method is new and requires practice or refinement, mice that are not used for any experiment (such as mice not needed for experiments from breeding, having the wrong genotype) or tissues not needed from collaborators experiments, will be used for practice.

Finally, we always endeavour to collect and share tissues that are not needed with collaborators or for future potential analysis (for example, collecting spine from transgenic mice that only hind limbs might be initially needed, providing tissues for undergraduate students and providing preliminary data for future grants).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are the most appropriate species due to techniques available to modify their genome. In addition, osteoarthritis development in our models is well described and resembles closely human pathology.

Tail tipping for genotyping has long been overtaken by ear clip just after weaning.

The models of OA used in this project are all non-invasive; this in itself is an important refinement for the animal, and also scientifically (limiting confounding factors such as wound healing, infection due to surgery, no micro-surgical skills required). The loading regimen used in this programme was devised to limit severe injuries that have been described and used by other groups (including joint dislocations, bone fractures).

For ageing animals, we have in place a scoring system for welfare assessment and determination of humane end-points for ageing animals.

The use of inducible Cre systems allows to “activate” gene deletion or overexpression to specific ages; thus to avoid any possible effects of developmental issues, activation will be induced after weaning (Fibrillin-1 deletion is embryonic lethal, but animals remain healthy if deletion is achieved after weaning). In addition, targeting to specific tissues can also limit severe detrimental effects on the animals health. For example, fibrillin-1 deletion from the cardiovascular system leads to early death, we therefore use musculoskeletal-specific Cre.

When possible, WT females are used for breeding: this ensures that the females are less likely to encounter issues during pregnancy that may be linked with gene mutation.

Why can’t you use animals that are less sentient?

OA is a slow progressive disease taking months to develop, and seen in adult and old individuals. Therefore adult and aged mice are necessary for some of our aims.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always try to provide alternatives in route of administration of substances: for example through food or drinking water rather than injection. We provide analgesic following surgery and increased monitoring and weight measurement to ensure animals are recovering following procedures.

Experiments are terminated when new unexpected phenotype occurs and are affecting the animals' welfare, always in consultation with our local vet

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In the scientific community that work on animals , we all follow as the Animals (Scientific Procedures) Act 1986 (ASPA) and guidance. New development that focus on " lessons learnt" disseminated by the NC3R website, LASA recommended maximum volume of administered substances and guidelines for blood removal as well as intervals between imaging sessions (LASA appendices A-C).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As an animal user and a BSU user representative, I receive regular updates from the NC3R through emails and through meetings held locally by the NC3R regional programme manager. In addition, the BSU is very informed and willing to improve animal welfare whenever possible and regularly discusses possible adjustments.

60. Understanding genetic and diet effects on the productivity and environmental footprint of dairy cattle.

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

Genetic selection, Feeding systems, Environmental footprint, Microbiomes, Animal health and welfare

Animal types	Life stages
Cattle	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims are: to identify effects of long-term selection for yields of milk fat and protein on a series of measures of production and technical efficiency of dairy cows; to identify interactions with different feeding/management systems; and describe mechanisms explaining these effects.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The study will be an important data resource for development of many components of new breeding indices, underpinning national and international dairy breeding programmes. Genetic selection and altered feeding have both been used to increase the productivity of Holstein cows. These tools will continue to be used as farmers seek to improve health and welfare of cows, as well as reduce the environmental footprint of milk production. This work meets the need to monitor a broad range of effects of genetic selection for milk output (milk production and composition; cow health and welfare; carbon footprint and other environmental consequences). We also need to understand how genetic selection interacts with cow management and feeding, as well as the mechanisms underpinning these effects and interactions.

What outputs do you think you will see at the end of this project?

Database information for use by the animal breeding community to develop new selection indices. The project will provide data from controlled systems which will be used to improve selection in the national herd.

More efficient, resilient and profitable dairy cows. Improved selection will be combined with refinement of systems to improve the sustainability of dairy farming systems with improved animal health and reduced environmental impact.

Publications about the interactions between genetic selection lines and feeding systems.

Publications about the role of microbiomes in mediating the effects and interactions of animal genetics on performance and health of dairy cattle.

Who or what will benefit from these outputs, and how?

All recording from the study will go into a database, which has comprehensive records dating back to the initiation of selection lines. This is an invaluable resource for animal breeders developing new selection lines and needing to understand interactions between different traits. By this means the work contributes directly to genetic advances in the national and international herd, with dissemination through genetic material (semen and embryos). Genetic effects are by nature permanent and additive, so guaranteed in both the short- and longer-terms.

The work also contributes to the design of new dairy production systems to improve the environmental footprint of dairying, as well as the health and welfare of cows. The work to understand these effects through measuring interactions between genetic selection lines and feeding systems, as well as understanding mechanisms behind these effects will mostly be disseminated to the global scientific community through scientific publications. The study also provides an excellent platform for dissemination of new concepts and practices to farmers. Previous examples include dissemination of the use of multi-trait selection indices (PLI), adoption of genomic breeding values, as well as work on body reserves and cow fertility.

How will you look to maximise the outputs of this work?

This work will inform the national breeding programme for Holstein cattle. We are continually looking for collaborations and partners to draw on the study resource for other purposes – utilising other techniques and expertise in the UK and globally to make use of data/samples collected in the study. In a recent example, work looking at lameness by scanning feet of culled cows benefitted from our extensive records of locomotion scores and hoof trimming across the lifetime of the cows.

Species and numbers of animals expected to be used

- Cattle: 600 – animals enter the study as young calves and remain under recording until 3 lactations are complete or they leave the herd for other reasons.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We have monitored animals from birth up to the end of the third lactation for many years, providing a unique catalogue of genetic progress. Recording has been focused on the first three lactations, with some monitoring of growth of calves and youngstock. We must continue the recording during lactations 1 to 3 and plan to increase the amount of recording from calves and youngstock, which is a major information gap. This has become possible because of (1) the availability of non-intrusive systems for recording the intakes and weights of calves, and (2) funding to study the establishment and effects of microbiomes at various critical sites of the ruminant body (upper respiratory tract; digestive tract; skin).

Typically, what will be done to an animal used in your project?

We will continue to maintain the study herd with approx. 200 milking cows plus the calves and youngstock replacements required to maintain the herd. We will continue the two selection lines (selected for either national average or highest possible fat + protein yield). These cows will be managed on one of two feeding systems in a 2 x 2 factorial arrangement of genetic lines and diets. One feeding management will be close to typical moderate energy feeding levels achieved on many commercial farms, whilst the other will feed higher levels of concentrates typical of the most intensive commercial systems. We will incorporate consideration of the embedded carbon footprint of diets into this design.

Ongoing recording for milking cows will include: feed intakes, water consumption, milk yield and composition, weight and body condition score, locomotion scores, oestrus and fertility records, activity monitored using commercial animal-mounted sensors, health events and veterinary treatments.

To avoid compromising the genetic experiment by treating different genetic lines differently, we will ensure that any procedures that involve handling/sampling animals, as opposed to non-intrusive monitoring, will be conducted on all animals within a cohort:

- For all animals - blood samples will be taken from a superficial vein, according to Home Office guidance on volumes – aliquots will be used for genotyping and others archived.

- For one cohort of heifer calves (100 calves over a 12-month period) we will also collect samples (faeces; swabbing of upper respiratory tract; naso-gastric or oro-gastric sampling of rumen contents; swabbing of inguinal skin) for microbiome and 'omics' analysis according to the following plan:

- At birth, 1 week and 3 weeks (upper respiratory tract and faeces)

- 2 months and 8 months (all sites)

- At mating, before calving and 30 and 100 days in milk (rumen and skin sites).

What are the expected impacts and/or adverse effects for the animals during your project?

Animals are managed under normal farm conditions with no planned restriction on availability of feed or water. We will monitor the natural incidence of disease under normal farm conditions, but nothing in the study is designed to cause disease.

Animals may experience some mild transient pain during blood sampling.

Microbiome sampling procedures and associated handling may cause minor discomfort and short-lived anxiety. Our animal handling facilities and staff training in handling and sampling of animals is designed to minimise distress. We will continually investigate and utilise best practice in the collection of samples in the least intrusive way. Naso-gastric or oro-gastric sampling from young cattle is a new technique for us and we will seek advice from project partners who have previous experience. Animals which show adverse reactions to sampling procedures will be withdrawn from the study.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect severity to exceed 'mild' for any animal.

What will happen to animals at the end of this project?

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The basis of this research is to collect and analyse data from actual dairy farming systems, these data cannot be obtained without animals in the different systems to provide the background data. These data will then be used to develop and improve existing models to obtain modelled outputs from a range of similar systems with different milk production levels. Animals are also required to link the genomic information with phenotypic information on animal performance, intake and health, as well as with the development and maintenance of microbiomes in the digestive tract, respiratory tract and skin surfaces. Alternatives to blood sampling to obtain genetic information are continually reviewed but to date no suitable alternative has been found to provide accurate longitudinal data.

Which non-animal alternatives did you consider for use in this project?

Modelling

Why were they not suitable?

Cow genotypes continue to change and so we need to maintain this monitoring of genetic lines to keep information relevant.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

With approximately 200 milking cows on the study, we expect around 100 heifer calves each year (5 years) and have previously reared all as potential replacements. With the availability of sexed semen, this calculation will be reviewed – we may have many more heifer calves born than are needed to provide enough replacements and, in that situation, will select a proportion for the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Each lactating group (system x genetic merit) will consist of 50 cows, the group size will be kept at approximately 50 by replacing animals which are either culled, in normal farm practice or are replaced at the end of 3rd lactation. At the end of the third lactation the cows are returned to the main herd.

Keeping cows on the study for 3 lactations is a balance to provide enough data for the systems component of the study and allow for a controlled entry of animals with improved genetic merit. Previous systems work has shown that 50 animals is the minimum to represent a farm system, current average herd size is 180 cows. During the 3rd lactation there is an average of 10 animals / system which in discussion with a statistician was considered to be the minimum number to obtain reliable data, especially on health and fertility.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. Our database contains comprehensive data from many years of work with these selection lines. It ensures that the animal breeding community can make use of these data.

2. We endeavour to share samples from these animals for use in projects designed by others – recent examples include lameness work using feet from cull cows; provision of milk samples for work on immune function and micronutrients; provision of milk samples for commercial development of calibrations for productivity and health traits using infra-red milk analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Sampling from the digestive tract involves per rectum sampling of faeces – we need to be confident that faeces were from a particular animal and not contaminated so cannot collect from the pen floor.

Sampling of rumen contents will use a naso-gastric or oro-gastric sampling tube method, which avoids the need for rumen fistulation. We cannot collect these samples after slaughter because we need sequential samples from the same animal, and also need subsequent health and performance records in order to explore relationships.

Why can’t you use animals that are less sentient?

Our research questions are specific to dairy cattle of specific ages – establishment and maintenance of microbiomes in calves; effects of genetic and diet on production and health during the transition period and established lactation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Having a dairy systems approach is integral to the study as the scientific questions relate to dairy production. All animals will be kept in designated Home Office compliant buildings with individual feeding, however the day-to-day management routines are similar to normal farm practice and veterinary attention will be sought for any animals which are ill.

If veterinary advice is that the animals need individual attention, they will be moved to a suitable area away from the main study group. If only for a short time (<4 weeks) then they will return to the study group. If removed for more than 4 weeks, or veterinary surgeon advises that they should not return to the study group then the animal will be permanently taken off the study. If >10% of the animals are removed from the study at any one time, the whole system will be reviewed and amendments made.

Animals which become distressed during any sampling procedures will be taken off study, under the guidance of the NACWO.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Home Office Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes.

Published guidance on blood sampling volumes, such as: Wolfensohn, S. and Lloyd, M. (2003). Handbook of Laboratory Animal Management and Welfare, 3rd Edition.

ARRIVE Guidelines (Animal Research: Reporting of In Vivo Experiments).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through ongoing literature review, we will maintain awareness of less-invasive techniques to study rumen function (such as analysis of urine, milk or breath)..

61. Investigation of drug resistance mechanisms in haematological malignancies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Leukaemic stem cells, Therapy resistance, Autophagy, Metabolism

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall purpose of this project is to gain a greater knowledge of drug resistance mechanisms that occur in the different types of leukaemia. We will investigate this using cell culture and animal experiments. Leukaemia describes four main subtypes, acute lymphoblastic leukaemia (ALL), chronic lymphoblastic leukaemia (CLL), acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML), as well as a number of less common types. Although we focus on targeting stem cells in CML, we have crossover projects in other leukaemia types, such as Philadelphia chromosome (Ph)-negative myeloproliferative neoplasm (MPN), AML and ALL. Some leukaemia subtypes behave similarly and research in one subtype naturally crosses over into the other subtypes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite decades of research and increased knowledge about how blood is made, blood cancers remain generally incurable. Between 2015 and 2017 there were 10,084 recorded new cases of leukaemia in the UK, and there has been a 17% increase in all types of leukaemia (AML, ALL, CML, CLL) since the early 1990s (CRUK statistics). Leukaemias are organised in a hierarchy (a pyramid- structure) with leukaemic stem cells (LSCs) at its top. These "originator" cells, called stem cells, are both in healthy individuals and in people with leukaemia responsible for the production of blood cells. However, only in people with leukaemia do the blood stem cells have defects. The LSCs are of interest since current drugs do not target them. New ways to study LSC biology are urgently needed which will help develop improved treatments.

What outputs do you think you will see at the end of this project?

Throughout these studies we will use animal experiments in combination with computer modelling and computer assisted drug discovery to create information libraries from leukaemia stem cells that are resistant to drug treatment. We will work closely with the pharmaceutical industry with the aim of:

Generating data leading to a better understanding of drug mechanisms in leukaemia cells to aid in the discovery of new drug targets.

Discovering new drug targets and/or novel drug combinations with existing therapies, which will assist doctors in the delivery of rationally designed clinical trials.

We aim to publish a number of scientific papers in open-access, high impact journals and present our findings at key conferences and workshops which will allow us to communicate our findings to the wider community.

Who or what will benefit from these outputs, and how?

Prior to this project we performed a drug discovery project on leukaemia cells in the lab looking for new drugs against both AML and CML cell lines. This project gave us a hit list of potential new drug candidates to explore more. The follow-up of some of these potential candidates, will be a large part of this project.

Short term benefits

A better understanding of leukaemia and the related diseases in this study. This will help both scientists in academia as well as in the pharmaceutical industry. Our research will be

shared globally in publications, conferences, seminars, the press, and social media that attract clinicians, scientists, nurses, allied health professionals.

Medium term benefits

Understanding the inner workings in leukaemia disease in its cell environment as well as in the whole animal will give us a more complete view of the disease. This will allow the creation of drugs that have a greater effect on diseased cells while having less impact on normal blood cells than current drugs.

Long Term Benefits

This project is using mouse models, human cell lines and primary patient samples, therefore the results will be of clinical relevance, so clinical trial organisations will benefit from the findings in our study.

Patients may benefit from appropriately tailored clinical trial design and drugs which are explored and suggested from our studies. Health organisations such as NHS and National Institute for Clinical Excellence may benefit from saving in the costs of cancer treatments

How will you look to maximise the outputs of this work?

The aim is to make all our information available to the wider community, both scientific and non- scientific via relevant publications. We also have ongoing collaborations with industry.

Species and numbers of animals expected to be used

- Mice: 32,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the various mouse models were developed to allow investigation of the blood system in the animal, in disease, as well as the normal state. Blood cancers, such as leukaemia, are so complex that mimicking this in cells in the lab alone is unfortunately not sufficient. Most of these experiments will be done on adult mice, as they have a more fully developed blood system. AML and CML generally happen in adults, and therefore adult mice will be more appropriate.

Typically, what will be done to an animal used in your project?

The project covers a broad range of techniques, but for 80% of the mice the process will be to irradiate them, inoculate (inject) them with either human or mouse leukaemic blood (stem) cells and follow how the disease gets worse over time by e.g., microscopy. Animals

will be routinely monitored to assess disease worsening, and 50% of the mice will receive drug treatment to assess the drug effects on the disease. Drug treatment will be administered by a combination of oral gavage, injection or by surgical implantation of a minipump for up to 4 weeks. Animals will be on study for up to 20 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

The health of animals will be routinely checked using a health scoring chart to make sure they do not get too ill and also for robust analysis of the mice across different cohorts (mouse groups).

Irradiation (adult mice only):

Irradiated mice will be more likely to get an infection after irradiation, because of their compromised immune system. They will be kept in special (barrier) caging and handled with clean (sterile) precautions to reduce the risk of infection. Advice will also be sought from the Named Veterinary Surgeon regarding any treatment required.

Irradiated mice will likely show weight loss and they will get soft foods to stop weight loss, as well as antibiotics to pre-empt infections.

Administration of cells or media:

The procedure will conform to current guidelines when administering substances (e.g. www.nc3rs.org.uk).

There is a risk that the mouse may develop an infection because of being inoculated (injected) with cells, particularly if the mouse has less ability to fight infections. This risk will be reduced by the clean (sterile) preparation of the cells and media and by regular, close monitoring of animals after injection. If animals show signs of ill health or distress more frequent monitoring will be introduced, advice will be sought from the Named Veterinary Surgeon if necessary, or the mouse will be humanely killed.

Injections carry a small risk of organ damage and peritonitis, and can also carry the risk of thrombosis and phlebitis. These risks can be minimised by using experienced licensees to perform these tasks. Mice observed to be experiencing discomfort as outlined will be humanely killed.

Surgery and Anaesthesia (General adverse effects):

Surgical procedures will be carried out aseptically. In the uncommon event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary Surgeon, such complications can be treated.

All animals are expected to make a rapid recovery from the anaesthetic within two hours. Uncommonly animals that do not recover or show signs of pain, distress or obvious ill

health will be humanely killed unless a programme of enhanced monitoring and care is instituted, and the animal fully recovers.

Any animal not fully recovered from the surgery within 24 hours (eating, drinking and return to normal behaviour) will be humanely killed.

In each experiment, it is always our aim to cause the least severity and stress possible for the animals while still gathering meaningful data.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (causing no more than momentary discomfort) - 60%

Moderate severity (discomfort lasting no longer than 72 hours on any occasion) - 40%

What will happen to animals at the end of this project?

Killed

Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although our initial work will always be done using cell lines in the lab (cell-based experiments), there is a limit to how well you can replicate the cell and disease response to e.g., chemotherapy, in a petri dish. This is particularly true for something as complex as the bone marrow (the home of leukaemia cells), where many different cells interact and work together. You cannot mimic this completely outside a living organism.

Which non-animal alternatives did you consider for use in this project?

There will always be a great number of e.g., toxicity studies and other drug treatment studies done in the lab with cells grown in culture, before moving on to animal experiments. If possible, we will use cell line and primary cell-based alternatives to animal testing. Our lab works with human patient cells from consenting donors to further test our research questions and drugs. This also reduces the number of mice experiments needed. As paraphrased from Duarte et al. 2018* “The precise composition and function of the bone marrow niche is still under intense investigation. It is thought to be regulated by a complex array of cell populations, including arteriolar and sinusoidal endothelial cells,

mesenchymal stem cells, perivascular stromal cells, osteoblasts, sympathetic neurons, non-myelinating Schwann cells, adipocytes, megakaryocytes and regulatory T cells".

This gives a good idea just how challenging it is to replicate this cell interplay in a lab system. People have attempted it with some measure of success (see e.g., Munaka et al. (2011) "A MICROFLUIDIC DEVICE TO MIMIC THE BONE MARROW MICROENVIRONMENT: REAL-TIME OBSERVATION OF

THE LEUKEMIC CELL BEHAVIOR" (conference abstract)). However, this is very challenging to reproduce, and even lower throughput than mouse experiments. In future even at its most advanced microfluidic devices are still highly unlikely to encompass every single cell type found in the actual leukaemic environment in the living organism. Nevertheless, we are continuously searching for viable lab-based alternatives that could approximate the in vivo situation sufficiently for our experiments.

*(The interplay of leukemia cells and the bone marrow microenvironment; Blood 131(14) Duarte, Hawkins and Lo Celso (2018))

Why were they not suitable?

Growth conditions in the body play a critical role in disease in both leukaemia and solid tumours. With the technology currently available to us we cannot duplicate these growth conditions in a petri dish in the lab.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals is the estimated total use for the 5 years that the project will run. Many animals are born with the incorrect genetic make-up for our research. This is one of the reasons for the large number of animals estimated, many of the animals will not undergo any procedures other than testing their suitability for our research. Our breeding colonies are monitored constantly and tailored to meet our experimental needs. Where possible we will have colonies that are homozygous for the alleles required for our research, thus minimising excess animals. We also try to minimise an excess of animals minimising numbers of controls. As not all mice in our experiments will develop disease, we may need to repeat some procedures and for this reason we estimate larger animal numbers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We discussed our experimental design with bio-informaticians, including the AWERB committee bio- statistician. Using specific software to assess mouse number requirements for our experiments. We are confident we are using an appropriate number of animals in each experiment. Blood sampling and non- invasive imaging techniques (for example MRI) will be carried out for studies to obtain as much data from a smaller number of animals. Sperm/embryos will be frozen from lines not immediately required for scientific studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Ongoing monitoring of a given cohort (mouse group) allows experiments to be stopped as soon as there is enough data (information), thus minimising suffering whilst obtaining meaningful and publishable results. When possible, control mouse cohorts can be shared across studies, to reduce mouse numbers required for individual studies. Blood sampling and non-invasive imaging techniques will be carried out for studies to obtain as much data smaller number of animals. Pilot experiments will be used to e.g., assess maximum tolerance and guide further experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a variety of mouse models in this project (mentioned in detail in protocol 3), all of which are specifically developed to allow better investigation of the disease, by being e.g., more susceptible to the human disease or have fluorescent markers that allow more straightforward investigation of processes within the organ(s) of interest. For example, NRGW-41 which has been reported to significantly enhance the percentage engraftment of human HSCs in the absence of irradiation. GFP-LC3 mice have fluorescent markers, which removes the need for administration of fluorescent tracers. The strains we will use allow more straightforward investigation of processes within the organ(s) of interest without the need for additional procedures. All these models were developed to cause the least pain, suffering or distress possible while providing us with valuable data on the disease of interest.

Why can’t you use animals that are less sentient?

Mice are still sufficiently closely related to human beings to adequately represent the human condition, whereas other model organisms more distantly related would not duplicate the disease sufficiently (on a genetic and phenotypic level). Most of these experiments will be done on adult mice, as they have a more fully developed blood system. AML and CML generally happen in adults, and therefore adult mice will be more appropriate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We always refer to the literature for adverse effects of a new chemical or drug and when a cohort (mouse group) is given a treatment for the first time, studies with a pilot-sized group (n=3-6) are carried out and closely monitored before extending to a larger cohort.

Animal monitoring, post-operative care, and pain management are an important part of all the procedures within the project. We use genetically engineered mice which exhibit the same genetic mutations in human cancer. None of our protocols exceed a moderate severity level. To minimise suffering all mice on procedure will be frequently monitored and humanely culled when showing signs of worsened health or other specified end-point is reached. All researchers working on this project will have specific training for each of our models used. All animals are housed in a dedicated facility proactive with environmental enrichment and the use of anaesthesia and analgesia under guidance from the vet is routine practice. Post-mortems are carried out to investigate any unexpected deaths.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all studies we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010) and ensure best working practice. We consult the NC3Rs guidelines and monitor refinement where such practices are published (NC3Rs website).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All our work is in observance to governmental guidelines and the facility we perform our experiments at is under constant scrutiny (by e.g., Named Veterinary Surgeons etc.) with the intent to adhere to newer guidance when these are published. We are also actively involved in the university's 3Rs Day, and there are a number of Culture of Care events organized throughout the year to ensure everyone stays up to date with current guidelines.

62. Imaging and treating tumours

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Detection, Hyperthermia, Imaging, Therapy

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to develop safer, more effective, solutions for the early detection and treatment of cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are several cancer types where existing medical treatments are not very effective. This may be due to inability to detect them early enough, surgical interventions having low success rates, or drug safety concerns limiting the dose that can be administered. Improvements in these areas have the potential to significantly increase patient survival and quality of life.

Nanoparticles are tiny spheres (~50-300 nm in diameter) made of an inert or biocompatible material. Being significantly larger than most drug molecules, their circulation and distribution behaviour in the body is very different. In particular they tend to be taken up and then retained by tumours. If an anti- cancer drug is attached to a nanoparticle it can be delivered to tumours far more effectively and specifically than otherwise. This useful property can be enhanced by engineering the nanoparticles' chemistry or by the application of external 'triggers' that change nanoparticle or tumour behaviour.

Various forms of clinical imaging are commonly used to diagnose cancer and examples include CT and MRI. It is possible to attach imaging 'labels' to nanoparticles, which enhance their contrast against normal tissues. This allows the tracking of nanoparticles in the body, gives information on where they go and when.

If nanoparticles carry both a drug and an imaging label, they are described as theranostic (therapeutic + diagnostic). This idea has already demonstrated huge potential to enhance the effectiveness of existing drug treatments. But to be of value to patients, these 'proof-of-principle' findings (often discovered in vitro) need to be developed and translated to the clinic. This includes testing against difficult to detect and treat cancer types, optimisation of methods and technologies, and assessing the safety, efficacy, and cost of the new approach.

These technologies promise to detect then treat cancers early in their development and hold the potential to save millions of lives. In our project we will development, test, and assess the efficacy of some of the best of these novel approaches.

What outputs do you think you will see at the end of this project?

In this project the team will develop novel healthcare technologies that will benefit humanity. Our aim is to develop and promote safe nanotechnologies that allow for early cancer detection, and then combine them with sonic or microwave activation technologies that are non-invasive, applied externally, and highly controllable. This allows for specifically tumour targeted drug delivery, reducing the amount of (often highly toxic) anti-cancer drug in blood circulation. Our studies are designed to collect new information and explore new materials that respond to ultrasound and to microwave energies, allowing the design of novel protocols to introduce these technologies to the clinic. The project will dissipate this new knowledge in the form of publications, communications in meetings, and collaboration with other researchers, as well as in the development of products that can be tested and used in humans.

Who or what will benefit from these outputs, and how?

The long-term beneficiary of this project are human patients with difficult-to-detect or difficult-to-treat cancers (for example, pancreas, intestine/colon, or brain. The team has developed technologies that have the potential to be rapidly translated and become products. During the last years, the team has demonstrated combinations of materials and

imaging/intervention technologies that have shown proof of therapeutic efficiency in xenograft models. In this project the team will focus to translate these findings to the clinic. The team collaborates with oncologists, radiologists, and equipment providers to rapidly bring these safe and efficient treatments to human use.

How will you look to maximise the outputs of this work?

The team is collaborating with radiologists, clinical oncologists, specialists in pancreatic cancer, brain oncologists, as well as paediatric brain tumour specialists. These clinicians have approached the team to collaborate and provide advice for the translation to the clinical phase. The team has published a book and will progress to organising meetings about these non-invasive treatments to communicate the findings to oncologists and publish in high impact factor journals. The team will make all findings from this project available to the scientific community and to patients' groups.

Species and numbers of animals expected to be used

- Mice: 2200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use adult mice for the tumour models. These mice will be either genetically altered to develop cancer or will be inoculated with cancerous material which develops into tumours in the subcutaneous fat. This is the only experimental approach that can realistically mimic tumours and their vasculature biology. The developed nanoparticles are generally administered into the blood stream and require a fully functional tumour blood vasculature in order to reach the cancer cells. Assessing the effect of applied interventions also requires an experimental model that is as close as possible to human cancers. No other models are currently available to perform this analysis and lower species (such as fish) are inappropriate for modelling a human disease.

The adult mice are used as they can develop tumours that reach a minimum size that can be used to assess therapeutic effect. Younger animals would have to carry a disproportional to their size tumour affecting their wellbeing

Typically, what will be done to an animal used in your project?

The animals used in this project are either: i) unmodified for use as reference/controls; ii) genetically altered to develop precancerous lesions; or iii) injected with cells that develop tumours subcutaneously. The animals will undergo a series of treatments which may include one or more repeats of: injection or oral administration of drugs or labelling materials, blood sampling, bioimaging using a variety of approaches (e.g., near infrared

florescence or MRI), and the case of subcutaneous tumour models, the application of non-ionising, non-invasive radiation provided by focused ultrasound or microwave antennas to affect the tumour response to the injected drugs. The mice will be carefully monitored to track their health, body weight, and (where possible) the size and conditions of tumours. The studies would normally continue until either a series of images/samples was complete, or a tumour has reached a pre-determined maximum size. The time frames of this are dependent on the aggressiveness of the tumour model but we have previously noted that treated animals can show inhibition of the tumour growth for 2 months (compared to 14 days of untreated animals).

What are the expected impacts and/or adverse effects for the animals during your project?

i) Genetically altered mice. These have been genetically modified to develop intestinal polyps. These need to develop to stage where the models are a reasonable mimic of the human lesion stage and so some animals may develop pain, weight loss, anaemia, or other clinical signs over a period of up to 5 days. These mice will be closely monitored and would be humanely killed they start to show signs beyond defined humane endpoints.

ii) Subcutaneously induced tumours and administration of anticancer agents or other therapeutics. The treatments may cause some weight loss (about 10-15%) for about 3 days post-treatment. Inoculated tumours will grow to certain maximum size (12 mm diameter over about 2-8 weeks) before termination and are not expected to cause any significant behaviour disruption or pain.

iii) Application of ultrasound or microwaves. A small percentage (5%) of animals may show localised swelling/inflammation, or mild pain for about 3 days after intervention. This will be controlled by the administration of analgesics.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

i) Mice that develop polyps will undergo protocols of breeding, induction with tamoxifen, and injection of substances. Protocols are of mild to moderate severity (50% of animals). From these 80% will experience mild and 20% moderate.

ii) Mice that will be inoculated with tumours subcutaneously will undergo protocols of treatment with substances and intervention. This is of moderate severity (75% of animals).

iii) Control mice will undergo protocols of mild severity, generally injection with substances and imaging.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To achieve the aim of the project, animals that develop tumours are required. Much of the mechanism of action is based on biology of tumour vascularity and how it influences nanomaterial distribution and responds to external interventions. It is not possible to effectively study tumour growth rate inhibitions, the uptake and display of labelled materials by intestinal polyps, or the effects of hyperthermia or cavitation on tumour uptake, outside of a living animal and there is no experimental model now that accurately mimics the tumour and vasculature.

Which non-animal alternatives did you consider for use in this project?

The team has developed an in vitro blood brain barrier glioma model and are currently investigating pancreatic tumour organoids to test some of the parameters (e.g., efficacy of drug when it is released from the nanocarriers). The team is also exploring in silico computational methods to estimate the nanoparticle concentration in the tumour vasculature.

Why were they not suitable?

In vitro methods currently lack formed blood vessels and hence the element of nanoparticle extravasation to the tumour, and its modification due to external intervention (such as hyperthermia or cavitation). This is a key mechanism we explore.

In silico methods are still very early stage. Since the interactions between tumour vasculature and applied interventions are not yet well understood, they cannot yet be realistically modelled.

These alternatives provide useful pilot or supporting information but are not yet a replacement for animal studies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The team has worked for more than 10 years with similar projects using the subcutaneous tumour model with techniques like ultrasound and microwave, as well as imaging and labelling of tumours.

We have extensive experience using these subcutaneous tumours and we estimate this number based on previous experience.

We are going to experiment with a GA mouse and for this we liaised with experts holding project licenses using this model and we assessed the breeding and the genotyped animals that will enter the protocols.

We have estimated the experimental groups that we will investigate in the next few years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have used the Experimental Design Assistant based on data we collect using similar experimental models or from published literature results.

We have discussed the hypotheses and the aims of the experiments and we introduced designs that are well aligned with the experiments we will perform.

Imaging is used for all mice bearing subcutaneous tumours so each animal is used for multiple time points and used as its own negative control (imaging with no treatment).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding of genetically altered strains will be carefully managed to ensure sufficient but not excessive numbers of animals are bred for use on subsequent protocols, whilst ensuring no genetic drift within the colonies.

Where possible, we will use the same animal to assess multiple scientific endpoints where it is compatible with good animal welfare in the context of the overall animal experience (e.g. use same animal for longitudinal assessment of tumour growth).

Pilot studies using limited number of mice to determine feasibility of new experiments will be carefully planned and performed.

We will have detailed consultation with others who have used the relevant protocols.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

1. Genetically altered mice that develop polyps. These models have been selected as sufficiently accurate mimics of human disease and there are no good alternatives to their use. Close monitoring of tumour development will ensure animal suffering is kept to a minimum.

ii) Mice that bear tumours subcutaneously. These models are a widely accepted approach for assessing and comparing the anti-tumour activity of different treatment protocols while balancing good mimicry of human disease with minimised animal suffering. The predetermined endpoints (20 % weight loss; 12 mm maximum tumour size) have been selected from experience and consultation points at which few animals can be expected to show significant adverse effects.

iii) Unmodified mice as controls. These are likely to show little adverse effects but are essential to allow proper comparison of the treated tumour groups.

Why can't you use animals that are less sentient?

The team focuses on the development of future products that can translated to the clinic. This requires study using models that are an acceptably realistic mimic of human disease, including the interaction of tumour biology with externally applied interventions. Less sentient species are not suitable models, and we require the use of mammals of a size compatible with pre-clinical imaging and intervention equipment. Adult mice are the best alignment with these requirements. Terminally anesthetised animals cannot be used in this project as the effects of the treatment will occur some days afterwards.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The team is closely monitoring animals post treatment - normally monitored once per day by the team and once per day by the staff at the facility. This intense monitoring allows noting down any adverse effect and these will be tracked using scoring sheets.

We follow the NC3R instructions and training for recognition and prevention of pain suffering and distress and will introduce a score sheet to monitor their wellbeing.

Non-aversive handling methods using tubes e.g. plexiglass will be used .

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow advice from the Br J Cancer Guidelines for the welfare and use of animals in cancer research. 2010 May 25;102(11):1555-77. doi: 10.1038/sj.bjc.6605642.

The NC3R welfare assessment EU5 will be followed by all members of the team and refreshers will be compulsory before experiments.

The website on mouse welfare will be used throughout the experiments http://www.mousewelfareterms.org/doku.php

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The team is following the NC3R webpage routinely and they receive notifications from the related social media. The team is regularly consulting with the experts in the College AWERB and NC3R. The team is also following news on organoids (and similar models) and is in close collaboration with experts that use in silico experimental design.

63. Novel nanomaterials as delivery vehicles for drug and cellular therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

grafting, nanomaterials, biodistribution, anti-microbials, cancer

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	adult
Chicken	embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Nanomaterials have been recognised as superior vehicles for small molecule drug delivery and ideal matrices for cell transplantation. They open up optimal temporal and positional control of drug or cell therapy application and, thus, improve patient treatment. Here, we aim to understand the basic properties of novel nanomaterials in animal models heavily utilised in biomedical drug discovery to help their development and demonstrate their potential for translation in clinical development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Virtually all drugs have not only desired therapeutic effects, but also unwanted side-effects that are due to their off-target activity or toxicity, e.g., through metabolites. This makes it essential to keep their levels in the body within a defined therapeutic window, which might be larger or smaller depending on the specific drug's properties. Especially for drugs with a small therapeutic window or the need for very long treatment periods this might present a significant challenge to treatment success; may that be via reducing toxicity or improving pharmacokinetics as well as enhancing ease of application for the patient. Thus, finding better ways to apply current and future treatments is of high medical importance and has the potential to generate more effective and less toxic treatments, which will ultimately benefit human health.

Moreover, other therapeutics, such as biologics or cellular (auto) transplantation, might necessitate "packaging" solutions that allow delivery to the body in the first place. Recently, this has been demonstrated for the delivery of an RNA vaccine by others, but is also applicable for providing a cellular matrix for transplanting cells such as fat cells for restorative surgery.

Of note, such improved delivery forms can also benefit basic research and animal welfare, for example, through improving experimental drug delivery schedules to refine the routes or frequency of application. This approach can be utilised in various contexts, e.g., when delivering tamoxifen to induce transgene expression in mouse lines, a widely used method, or substrates for bio-imaging. A thorough understanding of less invasive methods compared to repeated oral gavage or injections has the potential to not only change the animals' experience but also make experiments more reliable and less variable.

In summary, it is important to characterise, understand and then expand the current toolset of nanomaterials. This application aims to address the current gap in knowledge and systematically improve our understanding.

What outputs do you think you will see at the end of this project?

A primary output of this project will be an increased understanding of the "structure activity relationship" of novel nanomaterials with their function but also with their behaviour in vivo. This will be disseminated in to the various academic and medical communities in form of publications and conference presentations of the groups involved in this project. Further outputs might include patentable nanomaterials for various purposes. Specifically, we expect to have screened and developed a number of novel nanoparticle entities to deliver drugs, e.g., for cancer chemotherapy or antibiotics, and have secured follow on funding to develop these towards clinical applications.

Furthermore, a number of Hydrogels will be tested with the expectation to be able to move effective formulations towards clinical development. Lastly, we expect to be able to deliver proof-of-concept evidence for transdermal delivery patches for 1-2 chemicals often used in experimental animal studies, e.g., to trigger transgene expression or for imaging purposes.

In addition to developing new health-care technology, we will endeavour to publish the novel materials and their properties in appropriate biomedical and chemistry focussed peer-reviewed journals. We expect that multiple publications will arise from each part of the project, not only reporting on the materials themselves, but also on the underlying, fundamental effects and interactions with physiological processes. Lastly, we expect to report on novel methodology enabled by transdermal delivery of compound to experimental animal models.

Who or what will benefit from these outputs, and how?

In the short-term (before project end or shortly thereafter), we hope to have established a pool of candidates that can be taken forward for further efficacy testing in disease models of cancer and bacterial infections, which we need to establish the pharmacology and toxicology parameters for in the first place.

In the long-term, we expect a real health benefit for the human population from the ability to deliver drugs and embed transplantation material in nanostructured materials. Our project would have at least delivered some of the vital information on how these materials behave in a biological context and at best pushed one or a number of them to the clinic for a direct patient benefit. In addition, we feel there is a good possibility that animal welfare could also benefit from our research into transdermal drug delivery patches for experimental animals.

How will you look to maximise the outputs of this work?

We have an extensive network of collaborators inside the UK and beyond that will benefit from the gain in knowledge in general and by sharing reagents and results. However, a number of current and future collaborators in the research organisation will also be benefiting from the knowledge and methodology base build in this project and will further develop. A number of proposals are under development that would further benefit from the knowledge we will acquire.

Part of the project is to develop further the transdermal delivery of substances in rodent and chick chorio-allantoic membrane (CAM) experimental models, which will also be disseminated through contacts we have established, for example, with another large contract research animal facility in the UK. Such largely non-invasive methods might hold a great advantage for recurrent doing of chemicals via oral gavage or other injection routes.

Species and numbers of animals expected to be used

- Mice: 3600
- Rats: 240
- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents (Mice and Rats)

In line with current practice, this project will use adult mice for the majority of the in vivo characterisation work and only choose rats where prior models have established an overwhelming consensus to do so, or so far unforeseen experimental difficulties necessitate this. Both species have been extensively studied in their physiological parameters and reactions to xenobiotics in general as well as nanomaterial in particular. In addition, these rodents have been well established as models which lead to conclusions relevant to humans, and there are a number of transgenic animals available that will enable a more detailed refined experimental approach.

Chicken (Embryos)

The chick chorio-allantoic membrane (CAM) model is well established model for tumour angiogenesis that has received renewed attention more recently as an ethically advantageous alternative to rodent tumour models. We are using it here to pre-screen compounds and nanomaterials, which will reduce the number of more sentient rodents to be used. It is thought that the chick CAM assay is painless, as the CAM is not innervated by the embryo and the early developmental stage.

Typically, what will be done to an animal used in your project?

Rodents (Mice and Rats)

A subgroup of transgenic animals will be used for breeding purposes or humanely killed only to supply cells or organs for analysis and transplant material.

A further subgroup of animals will be used for subcutaneous implantation of cells with or without a nanomaterial as extracellular matrix, and the growth of such cells will be monitored over time using imaging and/or callipers. The animals will then be killed, and tissues are taken for further analysis. We might use syngenic wild-type lines or immunocompromised animals for transplantation of human cells.

The majority of animals will be used for administration once or multiple times of nanomaterial and then imaged and/or sacrificed for further quantitation of the nanomaterials or their effects on the rodent.

Administration routes can be nasal, intraperitoneal, intravenous, oral or transcutaneous. A small portion of these animals will be grafted with subcutaneous tumours before the nanomaterials will be injected to study the uptake of these materials in tumour tissue.

Chicken (Embryos)

The fertilised chicken eggs will be allowed to develop for a maximum of 18 days (3 days before hatching), at which point the embryos will be euthanised using a Schedule 1 method. At specific points during the development, we will open the egg shell or completely remove the egg shell to graft tumour material or cells with or without a matrix. The resulting tumours will be imaged and or treated with formulations of drugs to study their efficacy on limiting tumour growth or metastasis.

What are the expected impacts and/or adverse effects for the animals during your project?

Rodents (Mice and Rats)

Most common will be transient adverse effects from injections or inoculation with tumour cells or other graft material. These might lead to pain at the injection site or some skin irritation but will only be of short duration. Further, only short-lasting effects on the general wellbeing might stem from general anaesthesia used for tumour inoculations, grafting and imaging. Orthotopic tumour inoculation requires a small (<1cm) incision that is associated with pain during the recovery period, and a small chance of wound breakdown or infection.

Moreover, there might be direct and unexpected toxic effects of the tested nanomaterials that impact on the well-being of the animal and would lead to Schedule 1 killing.

Chicken (Embryos)

Based on published data as well as our own experience with this model, up to 50% of embryos will not start or stop development before the end of the experiment. Some of these might be adverse effects due to procedures that were carried out but might also be related to a number of other factors starting with the genetic makeup and transport of the eggs before inclusion in the experiment. It is assumed that there is no pain reception in the developing embryo up to 15 days of development. We do not plan procedures on the eggs after day 15 of development apart from imaging, but critically need those additional days to, e.g., monitor growth of grafts or tumours to establish the effects of pharmacological interventions. Thus, we do not expect to induce significant pain or suffering during those 3 days, but gain important additional information. All embryos will be euthanised latest on day 18 days of development.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rodents (Mice and Rats)

Breeding animals: Most animals will have at most mild severity as we will not use mice with harmful transgenes. However, few (<5%) may suffer complications during birth, which would be mitigated by Schedule 1.

Administration of substances: There might be up to moderate pain in <10% of animals that will be injected because animals will be injected with compounds below their known level of toxicity.

Grafting of transplants and turmour inoculation: There might be up to moderate pain in <10% of animals in the animals grafted with cells.

Chicken (Embryos)

The chick CAM is not innervated, and it is considered that the embryo has no capacity for nociception to day 14 (e.g., Eckrich et al. doi: 10.1038/s41598-020-75660-y), which means that the majority of all procedures will have minimal impact on the animals as they happen before day 14, i.e., the severity is at most mild for the procedures. However, those embryos that will be monitored up to day 18 of development before Schedule 1 might experience discomfort from the previous treatment or growth of tumour material on their CAM.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The human body is a complex environment and we are aiming to model the interactions of novel materials with this complex and dynamic environment. Drug behaviour in the body is dependent on how the drug is absorbed into the body by, for example the gut in oral drugs, how it is distributed throughout the tissues and blood, and then metabolised and excreted via the kidney or in faeces (ADME). Processes that are being shaped by multiple organ systems. Toxicity of drugs to the body introduces yet further parameters at multiple levels and distributed throughout the body that need to be considered. As such, no alternative to animal models is available at this point.

Which non-animal alternatives did you consider for use in this project?

We have considered and are actively using in vitro methods (2D/3D cell culture) as well as replacing the use of rodents with less sentient chicken embryos.

Why were they not suitable?

The in vitro models are not yet fully recapitulating the complex environment generated by mammalian biology, but only aspects thereof that might be misleading on their own.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Where known, we will use the expected effect sizes of quantitative parameters to estimate group size, and consult with the relevant literature for appropriate experimental designs as well as take expert advice on trial design to optimize our experimental program. A general rational for our estimated overall animal numbers, divided by animal species, for the project over the next 5 years are as follows.

Mice

The number of mice includes those used for breeding (protocol 4), which will be mostly transgenic and those from commercial breeders where no transgenic animals are needed. While we do want to minimise this, we expect that a portion of animals will not be used in this programme due to wrong genotype and/or sex. We estimate a colony size of an average of 2 breeding pairs over 5 years. (1000 Mice)

Furthermore, we to test at least 50 nanomaterials in pharmacokinetic and biodistribution experiments with or without payload delivery (Protocol 1, 2000), as well as grafting studies including 20 novel matrix materials (Protocols 2, 600 mice).

Rats

The number of rats is based on testing nanoparticle distribution parameters and propterties of 2-3 selected formulations (Protocol 1). Futhermore, it is based on the assumption that we will test up to 2 different gels in addition to the current Matrigel control and a further no-matrix control with up to 3 different kinds of grafted cells in groups of up 10 animals (Protocol 2).

Chicken (embryos)

We assume that we conduct experiments with up to 40 eggs per experiment, for 20 weeks per year for 5 years. Based on our experience with the model, there is a high attrition rate of infertile, non-developing eggs that we account for by overestimating the needed eggs by 25%, which bring the total number to 5000.

Biodistribution and pharmacokinetics

The relative and/or absolute amount of nanomaterial will be assessed at multiple time-points after single or multiple application.

To achieve sufficiently rigorous results, we anticipate using around 100 mice.

Novel materials for grafting

Group size will be determined for growth of transplanted cells will be compared between novel matrices vs. "state of the art" matrigel over time. Using longitudinal designs with imaging will enable to improve data quality and reduce the number of animals needed to reach meaningful statistical comparison.

Chicken CAM

Based on previous experiments we estimate to require around 100 eggs per experimental group. This is determined not only by the effect size that are expected, but also by the significant drop-out rates.

While we have achieved comparable survival rates to day 14 compared to the literature, this might still be somewhat variable due to variables beyond our control (transport of eggs, seasonal variability, etc.), which means we have to plan with a certain contingency number in every experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will be guided by the PREPARE guidelines (Smith et al. doi: 10.1136/bmj.k760) to plan our experiments. Moreover, we took statistical advice on the design of the experiments by local statistician colleagues as well as an external company specialised in the chick CAM model. We used the best estimate of effect size based on previous experience with nanomaterials in our group or published literature to inform our group size calculations.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Firstly, we will monitor all transgenic breeding closely to prevent producing to many experimental animals. Regular project meetings of PILs involved in the research programme will guarantee the adequate breeding colony size, as well as appropriate planning of experiments to use the bred animals most efficiently.

Pilot studies will be performed to get good estimates of effect size and optimal dosing regimen. Should our program demonstrate that our estimates of effect size are wrong (either too big or too small) we shall re-perform the power calculations to ensure we arrive at a rigorous outcome. If it appears that the effect size is much smaller than anticipated for a particular experiment, we shall consider abandoning that part of the program.

We shall routinely harvest tissue to enable biochemical, genetic, cellular and histological/immunochemical analyses and share this tissue with our collaborators.

The in vivo imaging approach permits longitudinal data collection from the same animal/embryo and this reduces the number of animals/embryos required in the study as the repeated measurements will give greater statistical certainty and will allow study of various quantitative and qualitative parameters in one animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are a well-established model for studying the effects of novel chemical entities on the mammalian body and characterise their behaviour. There is a robust literature and wealth of potential data obviating the need to repeat past findings. Rodents in general are seen as a simplified and experimentally tractable model for far more complex mammals such as people.

Wherever possible, physiological testing of phenotypes will be achieved by using non-invasive methods such as: non-invasive imaging taking advantage of transgenic reporter mice to refine experimental approaches. More invasive procedures will be used only when there is good reason (from the non-invasive experiments) to expect mechanistic insight.

Throughout the course of the license, we will endeavour to refine procedures and approaches. In fact, a number of planned experiments are explicitly designed to improve current state-of-the-art for substance application in experimental animal models (e.g., patch application of tamoxifen and luciferase substrates).

We shall use ex vivo tissue as much as possible to study cellular mechanisms relating of polymer uptake, thus enabling future in vivo experiments to be appropriately designed to minimize animal usage.

Why can't you use animals that are less sentient?

We are trying to implement this where possible. For example, we have established the embryo chicken CAM model (up to day 14 of development) for a number of the different projects which will reduce the total number of rodents and, we firmly believe based on the literature and our own experience we can generate relevant and translatable data, which we will further validate in this programme of research.

Unfortunately, in some cases we need to be able to observe the chick embryos beyond day 14 of development because certain functionally important features are not present before (e.g., blood brain barrier is developing) and, for example, the length of time grafting of tumours, start of (repeat) treatment and day 14 is not sufficient to make valid conclusion on changes in tumour growth and, thus, interaction with or efficacy of the novel materials.

Moreover, we have established a number of in vitro assays to gather as much data about the chances of success for the nanomaterials before going in vivo.

However, the experiments proposed in this programme of work are not feasible in these models only as they are translational, i.e., designed to evaluate novel materials for future use in medicine.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have considerable experience with the in vivo imaging methods and have considerably refined them already over the past 2-3 years. We have implemented standardised post-operative monitoring methods to ensure that outcomes and adverse effects from our surgical procedures are carefully assessed. We use this information to optimise post-operative care and pain management.

We shall use rigorous aseptic techniques to protect against infection of the animals, may that be rodents but also the chicken eggs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We shall take careful note of advice from collaborators and researchers highly experienced in the various methodologies and models to ensure that we refine our experiments as much as possible. We shall follow the Laboratory Animal Science Association and NC3Rs guidance on best choice of models and refinements. For example, for drug administration we shall choose all dose volumes in accordance with the LASA guidelines for the administration of substances.

To ensure effective and rigorous reporting of our results. In publications, wherever possible, we shall aim to adhere to the ARRIVE guidelines which are recognised as

providing excellent transparent standards for reporting of research using animals and were developed by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow advice by the local NTCO, NACWO and NVS. In addition, we will consult the NC3R and Norecopa webpages and the relevant literature as well as attend scientific conferences where possible. Any relevant new refinements or advancements during the course of the licence will be implemented if possible.

64. Technical development of refined phenotyping assays.

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Refinement, Passive Phenotyping, Home Cage, Social interactions, Mouse Behaviour

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To develop new more refined, or to improve existing tests that look at the physical characteristics of an animal, that help understand the function of genes (Phenotyping tests). We aim to undertake this project in order to improve the quality and the reliability of data output, and/or enhance animal welfare.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Genetically altered (GA) mice are currently in widespread use in biological science and have been shown to be of great value in explaining and understanding the function of genes (the basic physical and functional units made up of DNA, that pass information from one generation to the next) and the ways in which the genes interact with

other molecules in the body to express their functions, in a wide variety of biological, physiological and pathological processes. Although many genomes have been sequenced, the function of many genes (estimated at approximately half) are poorly understood.

Over the last 5 years, the techniques based on specific molecules that help scientists study DNA and other proteins, for making changes in the genomes of model organisms like the mouse have become so sophisticated that it is now technically much easier to make multiple alterations in a genome including those mimicking variations in DNA of different individuals or different populations seen in human disease patients. The generation of ever more complex and/or relevant mouse models to be used in the study of genetic disease is driving the need for sophisticated and, importantly, quality controlled tests to properly describe the distinctive nature and features of gene function and generate relevant animal models which can be used for pre-clinical studies to develop new therapies. A phenotype is a set of observable characteristics of an individual, resulting from the interaction of its genotype with the environment, where as a genotype is the genetic constitution of an individual organism. This facility supports individual research laboratories to characterise the result of specific alterations in the genes for their animal models by performing quality controlled phenotyping tests which may be outside of the laboratories area of expertise or not available elsewhere. In this way we are contributing to the development of better preclinical animal models. At the centre of the management of these projects is the development of improved tests, the continued quality control of those tests and the delivery of biologically and clinically relevant data to scientists the world over who work in the field of biology and medicine (the international biomedical community).

The methods of conducting behavioural genetics have changed very little over the past 40 years, in that they rely on test batteries (or a group of tests), that aim to link the outcome from a standard out-of-cage test to a behavioural phenotype. However, in order to conclude that the differences in behaviour between GA mice and mice where no changes to the DNA have been made are due to the genetic differences, the experiments must be controlled for the effects of factors other than the intended change, in this case the change in DNA, that can impact the results of the experiment. Mouse behaviour is flexible, dynamic and adaptive and therefore is influenced by a variety of genetic and environmental factors such as motivation, interaction with the experimenter, experimental design, test order, testing time and environmental enrichment. Therefore, it is incumbent on us to continually strive to refine the existing standards in behavioural phenotyping and to improve animal welfare through improving the quality and robustness of the data obtained.

In recent years, the advances in technology have made it possible to build upon established tests that study the effect on the behaviour response of animals to specific situations, to develop more automated, long-term behavioural testing platforms. These tests enhance and add to the value to the data generated by each individual test not lasting longer than a few hours, thereby building a comprehensive insight into the effects of gene mutations on animal behaviour and neurological function over days and weeks instead of looking at smaller ‘snap-shots’ in time.

Similarly, measuring metabolism in the mouse is also very susceptible to experimental parameters including fasting times, feeding regimes and light cycles. Recent data has been published on the effects of controlled feed regimes, as opposed to animals having access to food all of the time, and how normal testing regimes such as long fasting periods may confound the experiments. Together, all of these factors strongly indicate the need to refine existing protocols to develop tests which are more relevant to human physiology, remove confounding factors such as short observation windows and experimenter bias and deliver more translatable preclinical models.

Through this project we aim to offer a step change in the way mouse behavioural phenotyping is conducted; by trying to remove sources other than the intended change in DNA, that could result in changes in the physical or behavioural characteristics of an animal in the experimental models, refine and improve the experience of the animal and adapt phenotyping tests to being more relevant to the study of human disease. To do this end we will specifically explore different feeding regimes, ways of training animals in their home cage setting as opposed to moving them into specialist equipment and attempt to replace treadmills with voluntary locomotion whilst measuring motor activity. In addition, we aim to also explore mouse Ultra Sonic Vocalisations (USVs) to better understand mouse social interactions within their home cage environments and whether this can be used as a measure of well- being.

Thus by aligning animal welfare with more robustly measurable home cage behaviours we aim to deliver better preclinical research with an improved potential to develop drugs and therapies for use in human beings.

What outputs do you think you will see at the end of this project?

New phenotyping tests that will be refinements on current tests. By the end of this license we aim to be able to disseminate and train other researchers to use at least 3-5 different ways and conditions under which an experiment is conducted including the expanded use of home-cage monitoring, ultrasonic vocalisations (USV) for welfare and phenotype detection and altered feeding regimes.

All validated tests will be published and presented at the appropriate forum. All software designed or statistical methods produced will be available on relevant data-sharing platforms.

Who or what will benefit from these outputs, and how?

The main benefits from this research will be refinement through the generation of robust data by a) capturing behaviours in a familiar, enriched environment b) capturing behaviour observations that are not confounded by novel apparatus (equipment that the mice have never seen before), or human experimenters, c) serving as a permanent digital record that can be used by the wider community and d) generating video and audio data that are amenable to automated analysis techniques thus allowing new analysis to be conducted retrospectively without the need to generate more data.

Within the timescale of this project we are aiming to develop testing which is accepted by the wider scientific community and which contributes relevant, comprehensive data to significant scientific discovery. To this end we would not only focus on the development of the new protocols but also on the dissemination to other labs. Where possible we would aim to make the new experimental design easy for others to implement in their own laboratories by making them adaptable and provide relevant training.

We also hope, through our developing training programmes and relationships with groups such as the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), laboratory animal science associations (LASA/FELASA/AAALAS) and Institute of Animal Technology (IAT) to encourage the inclusion of new technologies widely in UK and international animal facilities and will initially be working with partners in three other facilities to do so.

Ultimately, we aim to improve the experience of the animals undergoing testing whilst delivering a broad and comprehensive dataset.

How will you look to maximise the outputs of this work?

The data will be retained as a permanent digital record of the tests; the aim will be to share this data through seeking collaborations with expert scientists in the field of research who specialise in a very specific area in that field of research and can provide expert advice on the work we are doing. These could be experts in the field of mouse behaviours to medicine and computer data scientists. The data will be disseminated through publications and presentations at appropriate forums.

We aim that the newly refined and developed tests become part of the tests used for preclinical studies and provide more robust, relevant and comprehensive data for screening new therapeutics. In deed we already have ongoing projects with two drug-discovery teams who are exploring the use of Home Cage monitoring to look for more relevant, earlier, indications of disease phenotypes in their mouse models. We would build on this work by bench marking the new paradigms against standard models to encourage uptake with confidence by other labs.

Species and numbers of animals expected to be used

- Mice: 10,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is necessary to use mice for this study because the main objective of this project is to refine existing or develop new tests to refine behaviour phenotyping in mouse models of

metabolic and neurological conditions where non-animal alternatives are not appropriate and/or available. These refinements would need to be tested against the existing standards protocols to validate them as well as encourage uptake from the wider scientific community.

We are studying neonatal, juvenile, adult and aged mice due to the nature of the conditions being investigated. Metabolic and social phenotypes (Social phenotypes are characteristics that relate specifically to interactions with other animals) may be manifested from birth, however, there are currently very few methods that do not rely on a period of separation from the mother or singly housing for a period of time. The work outlined in the current project aims to refine these methods, in order to be able to tease out such early phenotypes without interfering interactions of individuals within a group, such as the litter or the another mouse co-housed in the home cage.

Similarly, in order to investigate some phenotypes, especially those related with learning and memory it would be necessary to age a portion of mice on this project to beyond 12 months of age. As expression of dementia related phenotypes is increased by ageing it is therefore necessary for us to validate the refinements in mature adults that have a fully developed central nervous system.

Typically, what will be done to an animal used in your project?

Approximately 80% mice will undergo a combination of phenotyping tests to develop, refine and validate either the neurological or the metabolic phenotyping assays. Each experiment will use a combination of tests over the life-span of the animals, most of which are non-invasive, a small number involved anaesthesia used for immobilisation for the insertion of transponders to identify or record readouts use radio frequency to take readings automatically and from a distance.

Typically, mice are then terminally anaesthetised and blood and tissue samples taken. All experiments are expected to end by 12 months of age, with the majority of mice being culled by 52 weeks of age.

For some tests it may be necessary to singly house and or have restricted access to food. The aim of the current project is to refine such the set of conditions under which an experiment is conducted so that learning and memory tests may be conducted under reduced access and timed feeding routines instead of restricted access that may last up to 18 hours a day. As well as modifying assays such that they may be conducted under group housed conditions in the familiar environment of the home cage.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetic alterations in the mice used in this project may lead to the development of neurological or metabolic phenotypes. It is expected that mice modelling delay and difficulty in developing communication social interactions with other mice, metabolic or

neurodegenerative conditions may have some adverse effects such as aggression, obesity, progressive loss of movement, coordination, memory and sensory input such as olfaction, sight and hearing.

Mice undergoing restricted feeding routines in order to use a food-reward for motivation will have reduced access to food. In mice undergoing cognitive testing this may be to reduce their body-weight to ~90 % of their free-feeding weight. Studies have shown that in some knock-in mouse models of dementia causing disease, food restriction increases survival, consistent with data for C57BL/6 mice in general. Thus we do not anticipate adverse welfare outcomes associated with food restriction. To improve the validity of our results we will monitor long-term effects of food restriction by assessment of blood glucose before and after food restriction and body mass composition.

For some tests mice will need to undergo anaesthesia (e.g. for the implantation of devices for remote monitoring). All anaesthetic use in mice carries a risk of mortality and a risk of pain (short-term and long- term), which may differ between genetically altered lines.

Blood sampling requires the use of restraint to ensure safe sampling in a controlled manner. This poses the risk of induction of a stress response. The use of assays which minimise the required sample volume will be used and larger volumes of blood will not be sampled when the mice are on restricted food intake protocols.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice on the mild breeding protocol are not expected to suffer any adverse effects and the vast majority will not reach the expected severity threshold (sub-threshold severity).

On the moderate breeding protocol, it is anticipated that any mice carrying the disease-causing phenotype could exhibit a moderate phenotype. Other genotypes will also be born from these crosses, so approximately 25% of the mice may suffer a moderate severity.

Mice on the behavioural phenotyping protocol are all expected to reach a moderate severity. This is partly due to the phenotype of the mice, in which the genetic alteration could lead to a moderate severity in around 50% of the mice (the other 50% being unaffected controls). However, all mice will reach a moderate severity because of a subset of the phenotyping tests causing moderate suffering, for example overnight fasting for blood sampling or food restriction for the purpose of motivation in food- rewarded tasks. Additionally, the combined effect of repeating mild tests over the life span of the animal, in order to validate the assay over the progression of disease, will lead to an overall moderate experience. This cumulative effect of repeating mild-phenotyping tests may also interact with genetic alterations used in this project to model neurological and metabolic

conditions. Whilst these moderate affects will be short lasting they will increase the maximum severity of all animals on this protocol to moderate.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The primary objectives of this project is to refine existing or develop new ways of conducting experiments to refine behaviour phenotyping in mouse models of metabolic and neurological conditions where non-animal alternatives are not appropriate and/or available.

Whilst it may be possible to develop some of these tests in other species, the purpose of this licence is to refine the tests currently carried out in mice. The reason for this is two-fold, firstly many researchers have existing mouse models that they are already testing with the current techniques, we hope to offer them refined improvements in order to benefit mouse welfare and reproducibility in existing models.

Secondly, for complex systems such as behaviour and metabolism, it is often necessary to use a mammalian species to understand genetic alterations in the context of the whole organism. Whilst some preliminary work could and should be done in cells, or using computer modelling or in other species such as drosophila or zebrafish, when it is necessary to move into mice we want to be able to offer the most refined procedures possible.

Which non-animal alternatives did you consider for use in this project?

We aim to refine existing phenotyping assays used in mouse models, however, we would also keep abreast of developments in the wider field to ensure that any techniques that may be better addressed via non-animal alternatives are not developed on this project through use of animal models. Where possible we would use data already generated to develop automated techniques, such as image analysis and pattern recognition and use animals for validation purposes. For example, the use of computer modelling to predict patterns within big data this is especially relevant to time dependent pattern of behaviours, where it is not necessary to generate new data for understanding what is 'the normal pattern' but compare new data generated in GA models to historic data to tease out 'anomalies' that may be indicative of a phenotype.

Why were they not suitable?

The aim of this project is to refine existing procedures in mice. There are circumstances when earlier work will be done in non-animal alternatives, but when research has progressed to the point where a whole organism is needed we want to provide the most refined procedures.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Current samples sizes are estimates based on previous data. As we are refining procedures it is difficult to be certain of the correct sample size, however previous data from tests is used to enter into statistical equations that help calculate how many animals are needed in an experiment) for refined protocols to give us sample sizes needed for individual tests. As we are also developing new test protocols, as a first pass a highly specialised set of calculations that helps scientists decide how many animals would be needed for an experiment, where no previous data is available (G power calculation) may be used to determine sample sizes needed for a desired effect size. Where there is no previous data for power equations, resource equations may also be used. In GA models for neurodegeneration that will be typically used in this project some phenotypes do not develop until mice are 12-18 months of age. In order to ensure we have sufficient sample size at later-time points it is necessary to account for a percentage of mice that may not complete the study rates of 25% (this includes culling of mice to avoid the phenotyping of lone-housed because of loss of cage-mates. Rehousing of females will be undertaken but will need to be included as a variable in subsequent analysis).

An example of a typical study would be, 12 mice per sex and per genotype, thus (2 (genotypes) x 12 (mice) x 2 (sexes)) =48 mice will be required for each experiment. We anticipate undertaking approximately 15 such experiments per year for every year of this project. At least 2-rounds of breeding will be required to generate the mice required for the experiments outlined. Thus, in total we estimate we will need to breed 4000 mice for this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power equation calculator will be used for group size calculations for the phenotyping experiments. In addition, attrition rate calculation (to account for a percentage of mice that may not complete the study, as explained above) will also be used to ensure sufficient power is maintained until the end of the longitudinal studies.

The combination of tests in each experiment will be designed to gather the most meaningful data in order to validate the refinement. Tests which can inform each other will be carried out on the same mouse to remove inter-animal variability (this is the differences between individual mice that can influence the results) and increase the power, thereby decreasing the overall sample size and the scientific utility of generated data. We will also phenotype over the life-span of the animal to get better data from a smaller number of mice and to prove that the new/refined set of experimental plans is as accurate or better at detecting the same experimental outcome as the test it replaces over the progression of disease.

Standard Operating Procedures (SOPs) have been written and used routinely for standard tests and will be written and version controlled for the developing and refined tests. Each iteration of the test in development will be linked to the appropriate version in order to keep track of the assay development and validation in order to inform future refinements. This standardises the way the data is collected and reduces the variability and therefore the sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding will be used to minimise the number of mice being produced for these studies. Genetically modified lines that have already been studied will be sourced from repositories (these are carefully managed stores where the DNA of GA mice is stored for future use), to avoid remaking of lines whenever possible. Any excess stock will be offered to other researchers to minimise wastage. Pilot studies will be undertaken to generate means and standard deviations for work using back-ground strains for which data is not available. Tissues sampled from the animals used in this project will be shared with other researchers and the data produced linked to that generated by the project, to maximise long-term utility.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The overall objective of this project is to refine existing or develop and validate new refined phenotyping assays for metabolic and neurological models. A number of tests on this project are proposed not for routine use but for validation and welfare monitoring, so that other laboratories may adopt these refinements with confidence. In addition, only lines that

have already been characterised through broad based phenotyping will be used to validate the refined or new assays, in order to avert obvious welfare concerns. As the project aims to investigate phenotypes from a much earlier age and over extended periods of time, the refined assays may themselves reveal hitherto unknown welfare concerns.

Test	Why is this the most refined method?
Ear Biopsy	Mice need to be clipped for identification and the same piece of tissue is used for genotyping. Genotyping protocols (protocols are a clearly defined set of steps that must be followed when conducting an experiment; here the experiment is genotyping, which is the process of investigating if the change to DNA has passed on to the pup from the parents or not) have been optimised to use this very small sample size for all types of genotyping carried out at the establishment. This is more refined than other methods such as tail biopsy.
Insertion of Transponder microchip	Mice need to be implanted with RFiD (Radio Frequency identifier) transponder or a telemetry microchip in order to acquire positional data in the home cage or passively acquire telemetric data such as body temperature. The protocol for subcutaneous implantation of microchips has been optimised to use general anaesthetic and a local anaesthetic in order to minimise stress and discomfort during and after the procedure. The procedure further uses surgical glue to seal the wound in order to prevent accidental damage and or loss of the microchip device.
SHIRPA	SHIRPA is an observational test that involves placing the mouse in an arena or a jar and looking for abnormalities. This is non-invasive and can provide a lot of phenotypic insulin tolerance tests). This test is more refined than other methods of measuring glucose which require surgery to implant catheters that remain inside the body for the animal's life time.
Standard Calorimetry	This is non-invasive in home cage like equipment. Bedding is provided and animal shelters are used when activity measurements are not needed.
Metabolic cages	Mice are housed for as short a time as possible, usually 20hours. This length of time is needed to collect a large amount of urine and assess overnight food and water consumption. Red houses are placed in the cages to provide shelter and respite from the grid floor.
Home cage monitoring	This is non-invasive and measured in the home cage. After initial insertion of a microchip this test involves no further pain, suffering or distress to the mouse. This generates large amounts of data with no adverse welfare effects.

Why can’t you use animals that are less sentient?

The main objective of this project is to refine existing or develop new non-invasive assays to refine behaviour phenotyping in mouse models of metabolic and neurological conditions where non-animal alternatives are not appropriate and/or available.

This work needs to be undertaken in an animal with an intact nervous system without anaesthesia because we need to measure how effective the refinements in phenotypic assays are when compared to the standard phenotyping tests.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all tests it is important that the animal has no additional stress, therefore mice are handled calmly and provided the time to get used to testing rooms as well as arenas where possible.

For all tests mice are only housed in modified cages or arenas for the minimum time needed to gather meaningful data. Mice undergoing phenotyping tests have increased monitoring and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests. Mice which have had anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. When general anaesthetics are necessary, the combinations with least adverse effects will be used, for example for all tests only inhalation anaesthetics will be used. Pain caused by withdrawal of blood from the tail vein is reduced by using local anaesthesia and pain from insertion of subcutaneous implantation of microchip transponders will be minimised by general anaesthesia and local anaesthetic.

This license involves no surgery.

The validation pipelines will be designed with consideration given to the overall experience of the mouse and the number of type of tests any one animal will go through.

The project is specifically aimed at refining existing standard paradigms for assays such as operant testing, where the animals are typically food restricted for 18 hours a day for 90 consecutive days. By refining this food restriction regimen from 18 hours to timed feeding, the duration of the restriction may be reduced by up to 12 hours by focusing on the times of day that the mice are most likely to feed and thus still be motivated to perform a task for a food reward.

A combination of technologies, such as passive observation using video image analysis and modification of the home cage to use smart hoppers that would be capable of dispensing and depriving access to food automatically on a predefined cycle would have the potential to revolutionise such paradigms.

Training for experiments where animals learn to associate tasks with reward within the home cage is another example where the welfare burden on a mouse may be reduced by removing the need to train mice in a novel environment and away from its cage mates for extended periods of time.

By attaching behaviours to ultrasonic vocalisations a whole new dimension to mouse behaviour may be opened, as vocal cues can be a real game changer for indications of socially deficit phenotypes as well as early indicators for aggressive phenotypes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from LASA guidelines.

The establishment has full AAALAC and ISO9001-2015 accreditation. To conform with these standards, we must work to a high level of quality control on all fronts including husbandry, phenotyping and administrative processes.

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress

ARRIVE guidelines will be followed at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will attend general 3R's (Replacement, Reduction and Refinement) symposiums in the UK and abroad over the course of this project. From these I may gather information on refined phenotyping techniques or housing and husbandry methods. Moreover, it is my intention to continue to present any techniques that we develop ourselves in posters and papers in the relevant scientific/animal care forums.

More specifically, members of the team at the establishment will attend specific conferences that focus on all aspects of the neurological and metabolic disease, from humans, to model organisms to in-vitro (experiments done outside the animal e.g. cell lines) and in-silico (computer modelling work such as artificial intelligence and computer learning). Any new developments which could impact these studies will be discussed with the teams at the establishment as well as experts in the field who may advise on this project.

65. Antibodies for research and clinical purposes

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Antibodies, research tools, clinical research, Tumour markers, immuno-based assays, diagnosis

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project will generate antibodies towards disease biomarkers, which have no/or poor antibodies available. These can be used as tools to support research and improve clinical standards.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Research advancement, clinical assays and therapeutics by in large are dependent on the presence of tools such as antibodies. Antibodies have been utilised as probes to help decipher biological and pathological pathways. Importantly, antibodies are key

components of immuno-assays currently employed to aid in disease diagnosis and diagnosis, monitor treatments and provide effective therapeutics.

What outputs do you think you will see at the end of this project?

We anticipate to generate between 100 to 200 new antibodies towards targets that otherwise have no available antibodies. Through the course of the licence, we have developed and will continue to develop high quality antibodies primarily to targets relating to human cancer tumour markers, immunological conditions and a wide spectrum of veterinary species where there is a palpable shortage of antibodies such as salmon and chicken. Outputs include; products that will forward research through publication in peer-reviewed journals, diagnostic assays to aid pathogen and disease detection, prognostic assays to asses health and treatments.

Who or what will benefit from these outputs, and how?

The short-term impact (>6 months) is to advance science, especially where there is a shortage of research tools. Antibodies enable scientists to better understand the function and roles played by the corresponding targets thus enabling scientists to put together the pieces of jigsaw of a particular disease. Under our licence we have demonstrated competence in generating antibodies towards potential tumour biomarkers for research and clinical applications. We developed a number of mAbs with prognostic potential towards colorectal cancer (e.g. CYP26A1, CYP26B1, BCLAF1) (see relevant publications below). Under the previous license we have also generated 200 antibodies towards a spectrum of veterinary species targeting key immune and health markers, supporting such industries in the process.

The long-term benefit (>3 years) is that antibodies are essential components of establishing immunoassays to monitor human and animal health, evaluate vaccine performance and diagnose infections all of which will improve human health and the welfare of farmed species. Antibodies were also developed towards key molecules involved in pathological pathways such as the oxysterol and brown fat pathways (see relevant publications).

How will you look to maximise the outputs of this work?

Through collaboration with multi-international centres, we have generated a substantial portfolio of antibodies to key markers in salmonids, stickleback, zebrafish, tilapia, chicken, horse, seabream, seabass and shark to be used as research tools and immune assays. All antibodies are made available to the scientific community, over 80 of which have been utilised in ongoing research and publications.

Examples of selected publications:

-Hu Y, Carpio Y, Scott C, Alnabulsi A, Alnabulsi A, Wang T, Liu F, Monte N, Wang T, Secombes CJ. (2019). Induction of IL-22 protein and IL-22-producing cells in rainbow trout Oncorhynchus mykiss. Developmental & Comparative Immunology.

- Veenstra KA, Wang T, Alnabulsi A, Douglas A, Russell KS, Tubbs L, Arous JB, Secombes CJ. (2017). Analysis of adipose tissue immune gene expression after vaccination of rainbow trout with adjuvanted bacterins reveals an association with side effects'. Molecular Immunology.

- Alnabulsi A, Swan R, Cash C, Alnabulsi A, Murray GI. (2017). The differential expression of omega-3 and omega-6 fatty acid metabolising enzymes in colorectal cancer and its prognostic significance.

British Journal of Cancer.

- Yoon S, Alnabulsi A, Bird S, Zou, & Secombes CJ. (2016). Analysis of interferon gamma protein expression in zebrafish (Danio rerio). Fish & Shellfish Immunology.

- Swan R, Alnabulsi A, Cash B, Alnabulsi A & Murray GI. (2016). Characterisation of the oxysterol metabolising enzyme pathway in mismatch repair proficient and deficient colorectal cancer. Oncotarget.

- Yoon S, Mitra S, Wyse C, Alnabulsi A, Zou J, Weerdenburg EM, van der Sar AM, Wang D, Secombes CJ, Bird S. (2015). First Demonstration of Antigen Induced Cytokine Expression by CD4-1+ Lymphocytes in a Poikilotherm: Studies in Zebrafish (Danio rerio). PLos One.

-Brown GT, Cash B, Alnabulsi A, Samuel LM, Murray GI (2015). The expression and prognostic significance of bcl2 associated transcription factor (BCLAF1) in rectal cancer following neoadjuvant therapy. Histopathology, 10.1111/his.12780.

Species and numbers of animals expected to be used

- Mice: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice (8-12 weeks old). Animals with active immune response is prerequisite to generating high quality antibodies. This project will use mouse (BALB/c) model to generate antibodies.

BALB/c mice are useful for research especially in immunology. BALB/c strains are "particularly well known for the production of plasma B cells on injection with suitable adjuvant," an important process for the production of monoclonal antibodies. In addition, we have an established protocol in place at the for the generation of murine monoclonal antibodies.

Typically, what will be done to an animal used in your project?

A primary immunisation procedure with an appropriate adjuvant “complete Freund adjuvant (CFA)” using an appropriate dose will be administered by subcutaneous injection. At intervals of typically 2-3 weeks, three booster injections in incomplete Freund adjuvant (IFA) will be administered subcutaneously. The final booster injection in saline is then administered intraperitoneally. Blood samples will be optionally taken after immunisation to monitor immune response. Volumes and frequencies for blood sample will be used following the Home Office guidelines. If volumes/frequencies will exceed the recommendations, they will be agreed in advance with the Home Office after consultation with the Named Veterinary Surgeons.

The number of procedures carried out per animal are 6. The duration of the experiments is 10-12 weeks. There are no surgical procedures.

What are the expected impacts and/or adverse effects for the animals during your project?

Post-injection: the injected area may be slightly distended within few hours following the procedure carried out. Animals will return to normal within a short period of time.

It was observed after the intraperitoneal injection that some animals (~20%) showed some adverse- effects (minor temporary adverse reaction which can be resolved by supplementing the animals with additional heat).

Granulomatous reactions at the site of injection may occur in a small percentage (<1%) of animals following injection with adjuvant, which could last up to few days. It is worth noting that granulomas seen in earlier studies did not progress to ulceration. Under this circumstance the animal is monitored, and no further procedures are carried out until the lesion has healed.

If weight loss in some animals becomes apparent the mice will be weighed on a regular basis and monitored.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is mild. The process is mild throughout the duration of the study.

It was observed after the intraperitoneal injection that some animals (~20%) showed some mild temporary discomfort.

Animals will return to normal within a short period of time.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Live animals have to be used since an active immune response is an essential prerequisite for the production of murine monoclonal antibodies.

Which non-animal alternatives did you consider for use in this project?

A significant amount of our work is carried out in silico and in vitro and only a limited part of the work is performed in vivo (20%) using animals. Moreover, we established a recombinant technology (prokaryotic expression system), the aim of which is to replace the use on non-animal models to generate antibodies.

Why were they not suitable?

At present, almost all of research antibodies including research immunoassays, diagnostic assays, serology-based assays, monitoring immunoassays and therapeutic antibodies, are generated from animal models. There are emerging technologies which includes phage display and recombinant methods making strides to provide non-animal alternatives, but they remain in the developmental stage and few years away from coming to fruition.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals to be used based on our previous work/records. Simply, the objective is to generate 100 to 200 antibodies in a 5-year project which requires ~150 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Reagent companies and research institutes traditionally use two to three mice to produce one mAb specific to the protein target under investigation. Multiple animals are used as a backup as it is possible that on some occasions 1 mouse will not mount an immune response towards the immunogen of interest. At the start of this project licence, we followed this regime but by optimising our protocols, we now use animal with multiple immunogen (3 different immunogens) thus generating multiple antibodies to different targets of interest with no additional adverse effects on the animal. Therefore, we have reduced animal use by at least 6 fold , which is a significant reduction. We anticipate that during the course of this licence we will reduce animal numbers further by increasing the number of immunogens used per animal with no increase in adverse effects to the mouse or concentration or immunisation steps.

As mentioned above, mAbs are the ideal choice in terms of animal cost as once they are developed, they provide an unlimited source of antibody. On the other hand, pAbs are limited in amount and once the antibody supply is exhausted further animals need to be used. Currently we generate only mAbs which reduce animal usage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The novel approach we employ (peptide-technology) has become more sophisticated as we have gathered more experimental data over the duration of the last two licences. This enabled us to generate an AI software to increase success rates in production of antibodies which subsequently reduce the number of animals used. The AI software is made available to the scientific community and were recently utilised in the CSO COVID-19 programme to assist in producing a serology-based diagnostic assay.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model in this project is mouse strain BALB/c. BALB/c mice strains are associated with the production of plasma B cells following the injection with an antigen, an important process for the production of monoclonal antibodies. In addition, this model has

been used for the purpose of producing antibodies for decades and there are well-established protocols in place for the generation of murine monoclonal antibodies.

The protocols are associated with mild severity levels and minimum stress.

All procedures are carried out by experienced staff members at the designated establishment who are experienced and competent to handle the animals and perform the procedures minimising animal harm.

Why can't you use animals that are less sentient?

To generate antibodies suitable for research applications and clinical settings, mice with active immune response is an essential prerequisite.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- It was observed after the intraperitoneal injection that some animals (~20%) showed minor temporary adverse reaction. Two steps were introduced; (1) animals were put into a heat cabinet for 2 hours to speed up recovery after injection. In order to reduce the side-effect antigen samples were diluted 4 times in saline solution.

- If weight loss in some animals becomes apparent the mice will be weighed on a regular basis and monitored in relation to its physical condition as well behaviour. Any mouse exceeding 10% weight loss would be humanely killed.

- Complete Freund adjuvant (CFA) and incomplete Freund adjuvant (IFA) were used throughout our previous work. FA has been shown to be very effective in terms of reliability and success particularly in relation to smaller compounds such as peptides and it is the standard adjuvant of choice in antibody production. Having said that we are aware of the presence of a number of alternative commercial adjuvants e.g. Sigma Gold. During the licence we plan to carefully monitor any new literature for the use of alternative adjuvants and to pilot some of these adjuvants ourselves by designing the appropriate experiment. Where Complete Freund adjuvant is used it will be used on one occasion only, for the primary immunisation.

In order to reduce the side-effect antigen samples were diluted 4 times in saline solution.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R's Experimental Design Assistant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Attending conferences and international meetings. Follow the latest ongoing research at NC3Rs.

66. Study and manipulation of the tumour immune microenvironment for cancer therapeutics

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer therapy, immune, antibodies, immuno-modulatory, tumour microenvironment

Animal types	Life stages
Mice	pregnant, adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aim to evaluate the basic understanding of the regulation of the immune response against cancer as well as the development of novel anti-cancer therapeutics.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The success of immune-oncology therapies like anti-CTLA-4 and anti-PD-1/PD-L1 has built great potential for novel cancer therapeutics and paved a new era for immuno-oncology, especially for patients with melanoma. Despite great promise, responses are

limited to a fraction of treated patients and, critically, the mechanisms underpinning response and resistance to therapy remain elusive.

Therefore, this program of work aims to understand how the immune response is regulated in general, and how different tumour microenvironment can affect immune function. The program will also aid in the development and evaluation of novel and more efficacious combinatorial therapeutic approaches useful in the treatment of cancer.

What outputs do you think you will see at the end of this project?

The overall goal of this project is to study and interfere with the interplay between the immune system, tumours and their particular microenvironment using mouse models of cancer that better mirror human malignancies. We have already developed several biotherapeutics and have tested their efficacy and effects on tumour killing in mouse models with two of them currently in clinical evaluation. Data generated from these studies will give insight and mechanistic details on their function and toxicity to scientists and clinicians via peer-reviewed journals. Due to the increase in the development of new line of biotherapeutics for cancer I therefore expect the demand to continue to grow over the next 5 years of this PPL in accordance with clinical need.

Expected outputs derived from this PPL:

Products:

Biotherapeutic antibodies. New information:

Understanding single or combinatorial treated of immunotherapy and tumour rejection. Although immunotherapy have been applied to several of clinical trials very little is actually understood about why resistance occurs and immune exhaustion. We aim to answer these important questions.

Publications and public engagement:

The data obtained from this work will be communicated by publications to scientists and scientific conferences.

Clinical Translation:

Some of the products with the best results will be translated to the clinic/tested on patient samples.

Who or what will benefit from these outputs, and how?

Data generated in this study will be used to develop the next generation of immunotherapeutic treatments for broad a range of cancer types, to investigate and refine the mechanisms that underpin current immunotherapies and to produce preclinical data to support Phases I-IV clinical trials.

Because we study multiple different immunotherapies at different stages of development some of our data will have short term impact and some other will see impact after completion of the project. In the short term our work will impact the scientific community as we will provide basic understanding of how this therapies affect the immune system, whilst in the long term we expect to have clinical impact that will benefit patients with cancer as we will be developing and choosing to move into the clinic the most successful treatments and combinations therapies.

How will you look to maximise the outputs of this work?

This project is a continuation of a previous 5 year project which has been highly successful in the production of several biotherapeutics and publications. The work has facilitated numerous new collaborations with experts within the immunotherapy communities as well as clinical translation. This project proposal will maintain these collaborations to ensure continuity between the old project and the new.

We will distribute our data through the most appropriate channels, such as by presentations at national and international meetings, publications and public engagement events.

Species and numbers of animals expected to be used

- Mice: 28100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate model species for this investigation as they share similar genetic and physical traits to humans. In addition, whilst they are relatively cheap to maintain and have a short life-span, they can be genetically manipulated to use to understand chronic diseases and therefore excellent models of cancer evolution. Since cancer is a predominantly an ageing disease (with some exceptions such as childhood cancers) we will be using young adult mice in this project. The project also requires the use of genetically modified mice strains where the mice have a compromised immune system to prevent the rejection of murine/human cell lines. It is important to biotherapeutic products in human cells so that they are safe for use in clinical practice.

Typically, what will be done to an animal used in your project?

Because we study the immune response to cancer in animal models, the most common procedure in our license is the injection of tumour cells to induce tumour formation. In most cases the injection of tumour cells will be done under the skin or into the blood stream of the animal (with a syringe) without the need of a surgical procedure. In some cases (for

example when studying brain tumour formation), the injection of tumour requires a short surgical procedure (up to 20 minutes per animal). All procedures (injections and surgery) are performed only by trained experts.

Once tumours have formed and reached a defined size the animals will be given either one dose or multiple doses of a therapy (cell or drug) to evaluate their impact in the immune system and potential ability to control tumour growth. Some therapies are given through an injection into the blood stream whilst others require injection under the skin or directly into the tumour.

To determine if a therapy has an impact on the tumour, we measure the growth of the tumours over a period of time that varies with the type of tumour but that ranges from 21 days to a few months). The methods used to measure the size of a tumour depend on whether the tumour is under the skin forming a visible tumour or whether these are located inside the animal (i.e. lung cancer or brain cancer). For tumours under the skin we can measure the length, width and depth of the tumour with a tool similar to a ruler, whilst for tumours located inside the animal we will use imaging techniques similar to those used in patients with cancer and which are considered to be 'non-invasive'

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that have been injected with tumours may in some rare cases experience weight loss. Animals will be monitored 2 to 3 times a week and if an animal experiences 10% weight loss we will monitor its weight daily. In the very rare event that an animal loses 15% weight, it will be humanely culled.

In very rare cases the therapy may induce ulceration at tumour site which can be a consequence of the immune system destroying the tumour. Ulcerative scoring, body condition and animal behaviour will assessed regularly.

Surgical procedures and cell injections can in very rare cases cause internal bleeding or vascular occlusion (occurs when blood is no longer able to pass through a blood vessel). Animals are assessed rigorously during procedures and directly after upon recovery. Any animal showing evidence of bleeding or vascular occlusion will be humanely culled immediately.

If any animal reaches 15% weight loss with at least one other clinical sign, body condition score >3/4 or exhibits any evidence of with 24 hours: neurological changes such as seizure/fitting, respiratory distress, marked piloerection with hunched posture, reduced mobility, pallor, persistent ocular/nasal discharge, or diarrhoea, the animal will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Non-invasive imaging and tumour volume measurements will be used to monitor tumour development and tumour response we have therefore refined our expected impacts/adverse effects as much as possible as we are able to identify small morphological changes in vivo prior to the onset of clinical signs, thereby using much milder disease.

All tumour models and injection routes are routinely used within the group and undergo constant refinement to keep the severity level and potential adverse effects as low as possible. The severity is maintained as moderate due to the need for tumour modelling, surgical implantation and non-invasive imaging.

Expected mortality: In previous experiments we observed a low (0-5%) mortality as a result of these experimental procedures (NB. mortality is mainly from cell injections rather than the experiments as a whole).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to investigate the potential of different biotherapeutics for cancer immunotherapy to target and kill cancer throughout the body. Immunotherapy is dependent on immune cell infiltration and function into tumour tissue which is highly affected by the tumour environment and which organ the tumour is situated in within the body. Cell assays cannot replicate all of the complexities of the solid tumour environment within a host making immunotherapy therapy unpredictable. This proposal aims to address this by applying imaging to monitor immunotherapy in mouse models of solid tumours.

Which non-animal alternatives did you consider for use in this project?

We use in vitro lab assays (examples include cytotoxicity assays; antibody binding assays; organoid and tumour explant models) and bioinformatics such as single cell-sequencing/RNA- sequencing/TCR(T cell receptor)-sequencing to enable us to identify the therapeutic potential of certain immunotherapies. Computation modelling will be used to observe whether certain cancer types will respond to this therapy prior to moving into a mouse tumour model system.

Why were they not suitable?

Although in vitro assays can replicate some of the events present in cancer they do not replicate the biological, chemical and mechanical properties of the body, organ or solid tumour tissue. To optimise biotherapeutic effects on immune cell function and infiltration to tumours their behaviour needs to be monitored within a host with underlying disease. For this reason there is no in vitro alternative to animals that we can consider. Therefore, the use of animal models is essential to understand the intricate interplay between tumour cells, the tumour microenvironment and the entire immune system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We estimate the numbers of animals we will use from data obtained from previous experiments or from the literature. Moreover, with the expansion onto other cancer types, means and standard deviations will be taken from preliminary pilot studies consisting of 3 to 5 animals. The experimental design of these preliminary pilot studies will include appropriate controls to allow the detection of the effect of change of individual variables. Appropriate statistical analysis methods are then applied to the data obtained to obtain our estimates.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All in vivo studies are designed with assistance from the NC3Rs Experimental Design Assistant.

Preliminary in vitro analysis and computational models will be used prior to animal experimental design to reduce the number of animals used. Controlling variability such as animal housing, drug dosage, use of transgenic and immunocompromised mice, age, sex will be taken into account to reduce number of animals and repetition of experimental design. For patient-derived xenografts, the human patient tumour viability will be checked to ensure tumour growth is achieved. Moreover, both flanks can be used to implant human patient tumour rather than single flank, reducing the number of mice used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In establishing a new tumour model, testing drug dosage or assessing a new single to combinatorial biotherapeutic therapy a small number of mice (3-5) will be used as a pilot study where the mice will be assessed and adjustments made to make sure that models

are robust with minimum severity before being applied to a full experimental cohort. This will reduce the total animal number and constantly refines our protocols.

Tissue, such as tumour, spleen, lymph nodes, blood and other organs will be taken for histology, high- dimensional flow cytometry /protein analysis, and ex vivo validation to ensure our therapeutic cells are within the tumour tissue and to confirm the therapeutic effect and off-target toxicity. We ensure that any tissues generated from experiments are archived and stored appropriately therefore ensuring that unnecessary repetition of experiments is not necessary.

As part of good laboratory practice, all experimental designs including data analysis will be approved by the PPL holder before the experiment can start, and a protocol record for each experimental animal has to be kept by the PIL holder which will include the description of the experimental steps, treatments, and animal monitoring.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate model species for this investigation as they share similar genetic and physical traits to humans. In addition, whilst they are relatively cheap to maintain and have a short life-span, they can be genetically manipulated to use to understand chronic diseases and therefore excellent models of cancer evolution.

The tumour models we have chosen are well documented and well validated to produce reliable results within a moderate severity band. The tumour inductions are short procedures, and therefore the animal recovery is rapid and no adverse effects are expected. Visible tumours will be measured by callipers.

Whereas internal tumours will be monitored and sized by rapid MRI, CT, BLI or ultrasound scans to generate growth curves. Due to this, tumour growth in all settings, will be closely monitored and will not be allowed to reach a point where it may cause any discomfort to the animal.

Why can’t you use animals that are less sentient?

As stated above the mouse is the most appropriate model species for this investigation as they are the lowest animals in the evolutionary tree in which suitable models of human cancer can be carried out.

Host microenvironmental changes during animal development will influence tumour growth and cell behaviour which will continuously alter until adult hood adding unknown variables to the data and may result in irreproducibile data being generated. Adult animals have reached full organ development and therefore removes this variable, also due to size of the animal proposed adult animals are necessary for the procedures required for tumour implantation, drug dosage injection and imaging to be conducted using refined and practised methods.

Tumour models will be generated in adult mice at ages 6 to 12 weeks to ensure tumour reproducibilty and cell therapy as our data has indicated that increased age effects tumour implantation, animal recovery and immune status in some animal strains. Where assessing whether aged immune system affects tumour growth and treatment, mice over 12 weeks will be used.

The aim of this project is to assess immunotherapy in tumour models which are conducted over a period of weeks not hours we are unable to use terminally anaesthetised animals to conduct all aspects of these experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical implantations will be carried out aseptically to reduces any risk to infection. Animals undergoing surgical procedures will receive peri-operative analgesia (pain relieving agents given before or after surgery). Animals will be monitored intensely after surgical procedures and after cell/tissue implantation to check for clinical signs. Directly after the procedure the animals will be constantly monitored until they have completely recovered from the anaesthesia. Animals will then be monitored for the first 20 minutes then after a few hours within the working day and first thing the next morning. If no clinical signs are shown and the wound shows no infection or bleeding then the animals will be monitored 2 to 3 times a week. Needles used for implantation of tumour cells will be sterilised prior to injection to prevent tracking of cells and the growth of ectopic tumours (tumours formed in abnormal sites) .For IV injections and for blood sampling, blood flow to superficial veins will be increased by warming the mice in an atmosphere of 30 C for no more than 10 minutes.

The procedures within this project are run routinely and in our experience animals recover rapidly after surgery and very rarely require further post-operative analgesia after 24 hours except in the case of suture failure where the wound has needed to be re-sutured (within 48 hours maximum after surgery or under NVS (Named Veterinary Surgeon) advice beyond that).

When assessing animals after surgery and tumour implantation the assessment of pain will be assessed using the “grimace scale” (a method of assessing the occurrence or severity of pain experienced by facial expression changes) and will form part of an animals clinical signs assessment. A post surgical/cell implantation monitoring sheet will be completed each time the animal is checked over the first 24 hours using the animals unique identifier code. The monitoring sheets are specific to each of the protocols and cover the expected clinical signs for that protocol.

Animals will be weighed and tumour volumes measured 2 to 3 times a week. Any animal that reaches 10% weight loss will be weighed daily to make sure that it does not exceed the 15% weight loss.

To maintain that animals are not fasted for longer than designated time frame and if food cannot be removed at the desire time we will make sure that a measured number of pellets will be left in the hopper.

Lastly, we will constantly refine our protocols during the 5 year duration being guided by our analysis developments and the support of the NACWO (Named Animal Care and Welfare Officer) and NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All in vivo studies are designed with assistance from the NC3Rs Experimental Design Assistant and will abide by the ARRIVE guidelines. Myself and my group regularly consult the NC3Rs resources available in conjunction with our establishments guidance, training and meetings.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All PPL holders must attend yearly 3Rs meetings. We are committed to the principles of the 3Rs and will continue to seek the support of local NC3Rs representatives.

67. Investigating tick-borne bunyavirus interactions with ticks and how these interactions affect transmission and pathogenesis of viruses in a small animal model

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Ticks, Bunyavirus, Disease, Transmission, Therapy

Animal types	Life stages
Mice	juvenile, adult, embryo, neonate, pregnant
Hamsters	juvenile, adult
Rabbits	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To study bunyavirus associated disease in mouse models.

To understand how the interactions between bunyaviruses and ticks contribute to the spread of viruses and the severity of the disease experienced by mammalian hosts.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ticks are small arachnids of the subclass Acari. They are external parasites and live by feeding off the blood of mammals, birds, reptiles and amphibians. Ticks are divided up into one of two families, the Ixodidae (hard ticks) and the Argasidae (soft ticks), however this proposal will only concern the use of Ixodidae ticks. Ticks have four stages to their lifecycle, namely egg, larva, nymph, and adult. Ixodid ticks generally have three hosts, taking at least a year to complete their lifecycle. Each life stage (instar) requires one blood meal on a suitable host to progress through the life stages. Larvae and nymphs feed on small rodents such as mice while adults preferentially feed upon larger mammals such as dogs, cattle/horse/deer and occasionally humans. Due to their blood feeding nature, ticks are responsible for spreading many diseases that affect both humans and other animal species.

The term “arbovirus” refers to a diverse grouping of viruses that are transmitted by arthropods such as mosquitoes or ticks. Many arboviruses such as dengue virus (DENV), Zika virus (ZIKV) and Rift Valley fever phlebovirus (RVFV) are transmitted by mosquitoes. However, other viruses such as tick-borne encephalitis virus (TBEV) and Crimean-Congo haemorrhagic fever orthonairovirus (CCHFV) are transmitted by ticks. Insects and ticks are classified into distinct subphyla of the arthropods, the Insecta and the Arachnida respectively. The adaptation of individual viruses to spread by specific arthropods has a profound influence on the global distribution of diseases they cause, rate of disease spread and the ways in which we may prevent future outbreaks.

In recent years, we have witnessed a global increase in the incidence of tick-borne diseases and their spread into naïve populations causing potentially fatal illnesses. Data published in 2019, by the United States Centres for Disease Control, show that reporting of tickborne disease has doubled from 22,527 cases in 2004 to 50,865 in 2019.

Viruses such as severe fever with thrombocytopenia syndrome (SFTSV) have recently emerged and been reported in several asian countries (China, Republic of Korea, Japan, Taiwan & Pakistan) in the last five years. Data reported by the Chinese Centres for Disease Control reveal that between 2010 and 2019, there were 13,824 cases of SFTS disease reported in mainland China, including 713 deaths (average yearly case fatality rate of 5.2%). However, case fatality rates have varied between reporting countries from 5.2% in China to 32.6% in the South Korea.

Other viruses such as Bhanja virus (BHAV), produce a febrile illness in humans with photophobia, vomiting, inflammation of the brain and slight or partial paralysis. While Uukuniemi virus (UUKV) only causes a mild 'influenza-like' illness in humans. Unlike SFTSV, both BHAV and UUKV have caused disease in Europe and have been detected in endemic tick species. Currently, the only preventative measure available to treat these diseases is to avoid tick bites.

Despite the clear impact of these viruses on human health, we lack knowledge on many aspects of the basic biology of these pathogens and their invertebrate hosts.

The molecular interactions of tick-borne viruses with the mammalian host are vastly understudied compared to other arboviruses transmitted by sand flies and mosquitoes, while interactions between tick-borne viruses and ticks have been barely investigated at the molecular level at all. Despite causing severe disease and having an increasing global incidence, there are currently few effective antiviral drugs or vaccine candidates available to treat diseases caused by infection with viruses transmitted by ticks. There is also a continuing need to understand how and why these viruses are only transmitted by certain

species of ticks and how the biology and lifecycle of the tick vector contributes to the disease severity experienced by the mammalian host.

Here, our aim is to use small animal models to investigate bunyavirus transmission and pathogenesis using relevant tick species reported to transmit the viruses in nature.

What outputs do you think you will see at the end of this project?

Tickborne disease is placing an increasing burden on human and animal health worldwide. Despite this increase, little work is conducted on how and why pathogens like viruses are transmitted by ticks to other mammals through their bite. We hope to gain new insights into the biological mechanisms of virus transmission from the arthropod vector (an organism that does not cause disease itself but which spreads infection by conveying viruses from one host to another) to its mammalian host. To achieve this, we will need a source of ticks to use for these studies. As obligate ectoparasites that feed on the blood of their hosts, ticks require animals to progress through their lifecycle. We hope that these protocols will establish robust systems to raise and maintain colonies of laboratory reared ticks.

We seek to understand the contribution of the arthropod bite to the severity and spread of a virus within the host animal. We would like to discover the contribution of tick saliva to severity of disease, how viruses are transmitted in a population of ticks feeding on the same animal, how long it takes an animal to become infected following tick bite and finally, how long does it take for an animal to pass the infection on to an unfed tick.

The results of our study will be disseminated in the form of publications, presentations at meetings, and through press releases and other social media platforms.

Who or what will benefit from these outputs, and how?

These research outputs will be published in leading interdisciplinary journals so that they can be used by a variety of research and health professionals. The short-term benefits will primarily be specific to scientific researchers investigating tick-borne viral diseases.

Medium to longer term beneficiaries will include other researchers studying a variety of tick-borne pathogens or scientists/industry partners that are looking to develop anti-tick vaccines or therapeutics using live ticks.

The overall impact and goal of such work is to reduce the global burden of tick-borne viral disease.

How will you look to maximise the outputs of this work?

We will have extensive collaborations with entomologists (studying insects), acarologists (studying mites and ticks) and virologists (studying viruses). We will disseminate new knowledge in the form of publications, presentations at meetings, and through press releases and other social media platforms.

Species and numbers of animals expected to be used

- Mice: 2000
- Hamsters: 150
- Rabbits: 30

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.
Explain why you are using these types of animals and your choice of life stages.

The types of animals to be studied in this licence (mouse, hamster & rabbit) have been chosen as they are natural hosts for ticks in the wild. Ticks feed on increasingly larger animals as they progress through their life stages, and this is reflected in the animal use described.

The reason for the use of a wide range of animals used is two-fold: Firstly, as ticks mature through their life stages, they feed on increasingly larger animals. Tick larvae will feed on small rodents such as mice, whereas adult ticks will feed on larger animals such as rabbits; Secondly, if we find that the laboratory ticks do not bite/feed upon on a particular species of animal, we will need an alternative species to conduct experiments with. Pilot studies will be conducted to determine which species are preferred by our tick colonies and in time the licence will be amended to remove the species that are not needed.

Furthermore, previous work conducted has determined genetically modified mice bred to be deficient in the interferon alpha/beta receptor (IFNAR-/-) to be a robust model for tick-borne viral disease. This will be important for the assessment of the contribution of tick bite and saliva to viral pathogenesis.

The choice of juvenile and adult life stages of the host animals is based on published reports describing their suitability for tick feeding to maintain healthy tick colonies.

Typically, what will be done to an animal used in your project?

Animals will be infested with a strictly controlled number of ticks to allow them to take a blood meal. In some cases, the ticks or animals may be previously infected with bunyaviruses.

Animals may be administered drugs, tick saliva, antibodies or viruses prior to, during or after tick feeding.

Typically animals will be on procedure for 30 days. However, mice receiving primary and/or booster inoculations with vaccine candidates prior to tick infestation will be on procedure for up to a maximum of 90 days.

At the end of all procedures the animals will be euthanised and tissues and organs will be harvested for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

Some procedures are associated with mild severity rating and animals are not expected to suffer above and beyond anything that would not normally be experienced through tick infestation in the wild. Ticks secrete components in their saliva to prevent the host animal feeling pain or generating an inflammatory/itch response associated with the biting event. Also, ticks only take a blood meal once each life stage (instar), therefore we do not expect an individual tick to bite the host animal multiple times. Animals will not be exposed to ticks

more than once, repeated exposure may be detrimental to the feeding tick due to the generation of an immunological response in the host animal.

We therefore do not believe tick infestation will cause anything above moderate distress. When animals are infected with viruses, either directly using a needle or through the bite of an infected tick, we expect the animal to experience weight loss, piloerection, hunching or tremoring. We do not expect any adverse signs in any animal to exceed moderate rating. Any animals showing 3 moderate signs, or any single severe sign will be humanely killed. Animals undergoing all procedures will be monitored carefully and regularly by the personal licence holder to minimise distress and suffering.

All substances (such as viruses, antibodies or immunomodulatory agents) will be administered at doses known to be non-toxic, based on the availability (in the literature or otherwise) of their toxicity profile, and also based on their in vitro cytotoxicity profile. Pilot studies will be used where previous data is unavailable.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most procedures to be carried out are associated with a mild severity rating. For animals (mice, hamster or rabbit) infested with ticks, we would expect 95% or more of animals to experience mild severity. The number of ticks applied to an animal will be calculated to ensure that animals never experience anaemia due to blood feeding.

Mice are the only species that will be used for infection studies. For mice that we have infected with viruses (either directly or using a previously infected tick), we estimate approximately 50% of mice to experience mild severity and 50% of mice to experience moderate severity.

We do not expect any animal to exceed a moderate rating. Any animals showing three or more moderate or any advanced signs will be humanely killed.

What will happen to animals at the end of this project?

- Killed Rehomed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ticks and other arthropods (an animal with an external skeleton that lacks a backbone) are ectoparasites (a parasite that lives on/in the skin) of mammals by nature. They require to feed on the blood of a host mammal for their survival and for the progression through each of the different life stages (larvae, nymph and adult). The mammalian host varies between different tick species and even between different life stages. For example, Ixodes ricinus

(the castor bean tick) will feed on small rodents such as mice, birds, rabbits, reptiles and bats during the larval and nymphal life stages, whereas adults feed on large mammals such as sheep, cattle, dogs, deer, humans, and horses.
Therefore, to raise large and healthy colonies of ticks it is preferable to allow them to feed as naturally as possible within a laboratory setting.
For the disease pathogenesis and transmission work, only living animals can exhibit the complex interactions between cells in tissues, the immune system and the virus, which together, help determine how the virus can spread in different tissues and organs, and how it causes the disease. Therefore, animal models that mimic human disease are crucial in studies designed to understand the disease process and how viruses are transmitted from the tick vector to the mammalian host. The viruses used in these studies cause illness in humans, these studies will not provide improvements to our understanding of animal disease.

Which non-animal alternatives did you consider for use in this project?

Several groups have developed artificial feeding systems for some species of ticks. These systems allow for a small number of ticks to bite through artificial (plastic or silicone) membranes placed over small chambers of animal blood. These chambers can then be spiked with virus to allow ticks to take a virus-infected blood meal. We will use such systems to infect ticks with viruses to study the replication kinetics and infection biology of the virus within the tick itself. These chambers only allow small numbers of ticks to be maintained in the laboratory and are not suitable to sustain a colony for research purposes.

Cell culture systems can be used to perform biological analysis of the viruses in question in the context of infection.

Why were they not suitable?

Thus far, it has not been possible to develop membranes thin enough to facilitate biting and feeding of larval ticks. This is due to the very small mouth parts present on the tick in this juvenile life stage. This process will therefore only be useful for the nymphal or adult life stages of some tick species. Other reasons for not adopting this system are the very low attachment and engorgement rates for feeding ticks in this artificial setting. Even with fresh blood samples, thin membranes and chemical stimuli (such as host pheromones), artificial feeding rates are not considered robust enough to support the establishment of tick colonies. There are still some intrinsic limitations to the technique, including rearing many ticks, feeding efficiency, and tick fitness.

While considerable progress in the in vitro tick feeding of Ixodes ricinus was made by the development of an artificial tick feeding system using silicone membranes the attachment and engorgement rate of ticks fed on artificial membranes is lower than ticks fed on experimental animals and artificially fed ticks showed a decreased ability to produce new offspring. Also, the addition of antibiotics to sterile blood meals alters the tick gut flora with unknown consequences for pathogen transmission.

For Haemaphysalis longicornis, the only reported artificial feeding systems involve the use of a membrane made from mouse skin not silicone.

For Rhipicephalus sanguineus, larvae and nymphal ticks were not able to bite through the silicone membrane due to their comparatively smaller mouth parts than I. ricinus and

adults were only fed on artificial membranes for experimental studies. The R. sanguineus ticks used in these studies were originally derived from a laboratory colony that had been fed for several generations on laboratory rabbits or laboratory-bred beagle dogs to be able to provide sufficient numbers to conduct the studies.

From an ability to cause disease and spread of viruses perspective, cell-based systems are useful in advancing our knowledge on the basic processes related to the molecular biology and replication of bunyaviruses, they do not mimic many aspects of the complex multicellular environment of tissues and organs of living animals. Transmission studies of arthropod-borne viruses can only be conducted in the presence of the arthropod (tick) and its host.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For hamsters and rabbits, numbers have been based on experimental usage of external collaborators over the past 3-5 years. Collaborators have maintained a medium-sized tick colony using these numbers.

Numbers of animals needed has been back calculated from the number of ticks required. Tick numbers predicted to be obtained from infesting one hamster with 2 adult ticks or one rabbit with 50 adult ticks (performed in one, single infestation event) would be ~30 adult ticks (hamster fed) or ~780 adult ticks (rabbit fed). Each life stage or feeding is calculated with a 50% success rate imposed at each stage of development (we predict this is the minimum success reasonably expected). We believe the numbers of animals used will be sufficient to sustain the population of ticks needed for these studies, along with supplementation from field caught ticks.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We sought advice from biostatisticians to determine the animal group sizing.

Data from previous cell culture and animal work also has informed experimental design. For example, the amount of virus administered or the pre-treatment of animals with anti-interferon (IFN) antibodies to permit infection with some viruses.

These prior data mean a reduction in the number of animals used for infection experiments can be achieved to resolve our scientific questions. This can be further refined as new data are obtained.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have factored in sharing of tissues where appropriate with our collaborators to minimise the number of animals in the study.

We will conduct pilot studies to ascertain the feeding preferences of the different life stages of the three tick species to be used in this study. If it is observed that one or more of the tick species do not feed appropriately on a given animal type, the number of those animals used will be reduced. In the event that no tick species bites a given animal species, that animal species will be removed from the project licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse, hamster or rabbit animal models to establish our tick colonies as these are representative species that Ixodid ticks feed on under natural conditions.

Mice will be administered with antibodies, drugs or attenuated virus vaccine candidates prior to, and/or after, infection with bunyaviruses.

Animals will be typically on procedure for up to 30 days.

At the end of all procedures the animals will be humanely killed, and tissue and organs will be harvested for analysis as described below.

All protocols are designed to cause the least pain and suffering to the animals.

Why can’t you use animals that are less sentient?

The animals chosen are the least sentient animals that are suitable for our studies. Ticks are ectoparasites that feed on the blood of the host animal. Different tick species and tick life stages feed on a range of animals from small rodents to larger animals such as rabbits. We aim to replicate the natural feeding and biting behaviours of the ticks in our colony by allowing them to feed on animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals will be monitored daily and will be humanely killed if necessary, following veterinary advice. We will use local AWERB and other guidelines for dosing and for blood sampling, establishing clear humane endpoints, monitoring animals frequently, not allowing infections to develop into severe illness.

We will impose strict limits on the number of ticks that can be applied to an animal (based on the amount of blood withdrawn) and limit an animal to only one round of tick biting.

Animals on procedure will be given housing enrichment and additional ‘treat’ food (e.g. baby food for mice, as necessary) to improve their welfare while infested.

All laboratory animals will be handled using non-aversive methods as this has shown to have a positive effect on scientific data due to a reduction in baseline stress and anxiety of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Home Office and NC3R guidelines and those published such as:

Carbone and Austin (2016) Pain and Laboratory Animals: Publication Practices for Better Data Reproducibility and Better Animal Welfare. PLoS ONE 11(5): e0155001. doi:10.1371/journal. pone.0155001.

Percie du Sert et al. (2020) The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 18(7): e3000410. https://doi.org/10.1371/journal. pbio.3000410.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Keeping abreast with any new information and guidelines through the NC3R website and through our animal facility. We will constantly liaise with the animal facility staff and attend relevant presentations from them to ensure that we are up to date with the information and that they are implemented.

68. Neural mechanisms of pain control

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Pain, Nociception, Affect, Neuromodulation, Sensation

Animal types	Life stages
Mice	juvenile, adult, pregnant
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify neural mechanisms generating the sensory and behavioural dimensions of acute and persistent pain and develop neuromodulation strategies to control pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Persistent pain is a major problem for patients, healthcare providers and society. Current treatments lack efficacy and can cause harm, as has occurred with the ‘opioid epidemic’ in which pain treatment has led to addiction resulting in many tens of thousands of deaths, particularly in the USA but also worldwide. There is a lack of effective treatments to alleviate persistent pain and therefore an urgent need to improve understanding of its underlying cause and identify novel interventions to mitigate it, such as harnessing the

brain's own pain-relieving process as part of a therapeutic strategy. The work conducted under this licence aims to advance understanding of the mechanisms responsible for persistent pain, identify new treatments and translate these into the clinical setting. The findings of the outlined studies will be of value to pain neuroscientists, patients, clinicians and industry.

What outputs do you think you will see at the end of this project?

The anticipated benefits of the study will be to advance understanding of:

The specific neural pathways within the CNS involved in pain perception and how these become altered in states of persistent pain.

Neuronal mechanisms of pain control and why they fail in persistent pain.

The mechanisms by which pain alters cognition.

In so doing the work aims to identify more effective ways of treating persistent pain and to progress these towards the clinical setting.

Who or what will benefit from these outputs, and how?

The chief beneficiaries of the study outputs will be the academic, clinical and industrial research communities, who will use the knowledge to drive future research, deploy new diagnostic approaches and to inform drug and therapeutic discovery strategies. Information generated will be published in high impact peer-reviewed journals (as well as on preprint servers like BioRxiv) and presented to appropriate audiences at national and international meetings. We will communicate our findings at public and patient engagement events and include patients as research partners during the development of our research strategies. We expect that our outputs in the long term will benefit pain patients and the wider society.

How will you look to maximise the outputs of this work?

We will continue to collaborate with academic research colleagues at both a national and international level to best exploit our research findings – the majority of our work involves international collaborations. We have plans in place to continue to work with industry partners in the pharmaceutical industry. We will disseminate our research through traditional peer reviewed publication and scientific meetings but will also publish our research (successful or otherwise) as open access pre-prints and will share the data via appropriate repositories. We will present to the public and at patient engagement events. We will protect any intellectual property so that it can be developed commercially and turned into treatments.

Species and numbers of animals expected to be used

- Mice: 2,400
- Rats: 2,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Rodents have been chosen for these studies because they have the least neurophysiological sensitivity as species within which pain-related behaviours that mirror aspects of human pain can be modelled. Rodents share many of the anatomical and physiological aspect of the human nervous system and consequently have been used extensively in similar studies worldwide. Non-mammalian species (e.g. arthropods and zebra fish) are not appropriate as they either lack many of the brain structures of interest or, if present, lack the organization and connectivity with higher centres that we seek to understand. Additionally, they have different neurochemical mediators and distinct receptors, so their pharmacology is often not directly comparable to human.

Both mice and rats will be used for the outlined studies. Mice offer the advantage of ease of genetic manipulation, which facilitate the study of specific populations of neurons. Rats on the other hand offer advantages for pain behavioural studies in that they are calmer, more inquisitive and are better able to train and perform in assays of cognitive functions. Additionally, their larger size offers advantages for some types of electrophysiological experiments

Typically, what will be done to an animal used in your project?

The majority of animals will undergo a surgical procedure, most commonly a small burr hole craniotomy, typically for injection of a viral vector or introduction of a fibre optic probe or electrode. In cases where viral transduction is required, animals are returned to a group housed environment for 2-4 weeks (to enable sufficient expression of effector proteins). Animals with implants will initially be singly housed until the skin around the implant is healed (2-3 days) before being returned to group housing. A subset of the animals (20%) will have sensitisation of an area (typically hindpaw) by either nerve injury or administration of an inflammatory agent to produce a validated model of neuropathic or inflammatory pain (lasting up to 12 weeks which is necessary to see the stable expression of the sensory and affective phenotype).

Animals having behavioural testing / neuronal recordings (30% of animals on the protocol) will have sessions of acclimatisation to behavioural apparatus. They will have tests of different sensory modalities (e.g. touch – with a fine filament, brush, heat - from an infrared illumination, cold - from an acetone drop) applied at different intensities until they show withdrawal responses. This sensory testing will not involve more than 48 stimuli per session, with no more than one test session per day. Across the duration of the experiment (maximum of 12 weeks) there will be no more than 20 sessions in total.
This will allow precise assessment of sensory function across several modalities and its response to interventions such as analgesics during a single testing session and how that may change with the development of a pain model. The animals may have activation or inhibition of neuronal circuits using pharmacological or optogenetic methods and the effect on nociceptive processing or pain associated behaviours will be assessed before, during and after the intervention.

Some of the animals (10%) will have their food intake limited to 90% of their normal intake. This is to motivate the performance (using food rewards - appetitive behaviour) on operant tasks to test affective bias and decision making. They will only be fasted for the period of

training and conduct of the assays. They will have free access to water. The typical length of behavioural training is 4 days of familiarisation followed by a maximum of 21 days of task acquisition. The upper length limit is 48 days of task acquisition/behaviour.

At the conclusion of the study the animals will be killed via a Schedule 1 or non-Schedule method as appropriate. Some animals (20%) will have recording of neuronal activity to assess the responses to application of sensory stimuli while under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals on this project will have recovery anaesthesia for surgical procedures (60%). The animals are expected to recover uneventfully from the general anaesthesia and surgery and to resume normal behaviour within 24 hours.

The food restriction regimes used are not expected to adversely affect the wellbeing of the animals.

Some animals will experience pain following induction of sensitisation by either nerve injury or administration of an inflammatory agent as part of an experimental pain model of human disease (10% of mice and 25% of rats). They will typically show a degree of gait alteration with less weight bearing and sporadic spontaneous foot lifts / attending to the sensitised paw. These animals will continue to interact socially, to be mobile and able to self care and are not expected to lose weight. The condition and welfare of these animals will be carefully monitored throughout to ensure that they do not exceed a moderate severity level. Any animal deemed likely to exceed that severity limit will be killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Rats
- Non recovery 20%
- Mild 10%
- Moderate 70%
- Mice
- Non recovery 35%
- Mild 25%
- Moderate 50%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Each of our objectives has at its core the study of pain, sensation and its regulation with a focus on identifying treatments with the potential for translation into a clinical setting. Pain is the product of integrative processing by many classes of neuron both peripheral and central. Even the somewhat simpler process of pain perception cannot be adequately represented in reductionist in vitro models nor in silico given the complexity of the cellular and molecular specialisation and the impact that the sensory neurones have upon much of the neuroaxis. The aim of this study is to integrative physiological processes (for example the transition from acute to chronic pain) that are controlled by the central nervous system and which cannot be adequately recapitulated in anything other than intact animals

Which non-animal alternatives did you consider for use in this project?

We considered computer modelling and recordings from cultured cells such as stem cell derived neurons as alternatives. We also considered the use of human models. We use these for some studies and these have advantages in being able to directly link the findings to pain percept. Several of our research objectives aim to improve the ability to record in humans in the future.

Why were they not suitable?

Computer modelling requires biological data to build informative models and make accurate predictions – at present there is no model of any aspect of the pain pathway that has any predictive validity. Cell cultures cannot be used to make inferences about pain as this requires an intact nervous system integrating information from many sources. Human studies have clear limits in the ability to isolate and control the experimental conditions (through exposure, insertion of recording and stimulating electrodes and the use of genetic and pharmacological tools to probe neuronal circuits) to definitively identify neuronal mechanisms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have considerable experience in the conduct of pain experiments and the use of stimulation paradigms, recording methods and behavioural assessment. We have used this information to estimate the numbers of animals needed. For the GM colonies this has been scaled to take account of whether the strain of animals will be homozygous or heterozygous for the gene of interest. For definitive pain studies, where an intervention is made to reduce a pain-like behaviour, we have taken a meaningful effect as being a 30% change in the response (based on IMMPACT guidance). For studies using new methods

or models we have used data from the literature. For novel interventions we will run pilot experiments and use the data to estimate the group size required for definitive studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will make extensive use of within animal controls which immediately halves the number of animals needed for a study and also reduces the variance so increasing the power to detect effects. We will use inbred strains of animals to further reduce variance. The methods we are developing such as multi-unit peripheral nerve recording will further reduce the need for animals as they have a greater yield of data than conventional recording approaches. We will use animals of either sex and will stratify on this variable in our analysis to identify any sex specific differences. We use standardised surgical approaches and follow defined protocols for the application of defined stimuli and behavioural testing.

We make use of the NC3Rs experimental design assistant when planning our investigations and making proposals to funders.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible we will use homozygote lines of GM mice. We will make use of pilot studies for any new experimental protocols. Tissue from animals will be used for in vitro experiments such as brain slice recordings or skin-nerve preparations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a number of well-established rodent pain models (such as nerve injury models of neuropathic pain, skin incision models of post-surgical pain and inflammatory models of mono-arthritis) that are closely comparable to aspects of human pain conditions with translational validity. We will make standardised assessments of the animal's sensory responses using carefully titrated stimuli and also make observations of their behaviour and decision making to assess the impact of pain on cognition. For studies of pain, information will be gained from experiments in non-sensitised animals, which will be used to inform the design of experiments in models of chronic pain. This approach will minimise the numbers of animals used. In all cases the least severe model, consistent with obtaining the necessary data, will be used. For each protocol we have clearly defined humane end points. For some animals we will assay their cognitive and behavioural responses and in these cases the period of sensitisation will be kept to the minimum that is scientifically meaningful while also ensuring that longitudinal observations yield the maximal amount of useful data.

Why can’t you use animals that are less sentient?

Rodents have been chosen for these studies because they constitute the least sentient species within which human pain-related behaviours can be modelled. Non-mammalian species (e.g. arthropods and zebra fish) are not appropriate as they either lack many of the brain structures of interest or, if present, lack the organization and connectivity with higher centres that we seek to understand. Additionally, they have different neurochemical mediators, ion channels and distinct receptors so their pharmacology and physiology is often not directly comparable to human. Rodents share many of the anatomical and physiological aspect of the human nervous system and consequently have been used extensively in similar studies in many other laboratories worldwide. We will make use of terminally anaesthetised experiments for some measurements – particularly electrophysiology. By combining experimental approaches using investigations wherever possible in reduced preparations e.g. brain slices to address some of the cellular and circuit level questions in combination with targeted genetic approaches we will be able to minimise the numbers of procedures and the burden on sentient animals. Additionally, we will only progress to assay interventions in behaving animals that we have shown to be effective in these ex vivo experiments on electrophysiological / histological measures of nociceptive sensitisation.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have refined and developed our approaches. The animals used in these studies will be acclimatised to the unit prior to any procedure. Surgery will be performed aseptically under general anaesthesia with appropriate post-surgical analgesia. Animals will be closely monitored and allowed to fully recover after surgery before any subsequent procedures (unless post-surgical pain is the variable of interest). The surgical procedures and implants for our chronic recording experiments have been refined over many years to minimize their severity and to reduce the numbers of animals required to meet our objectives. Animals will be group housed (whenever possible) with environmental enrichment. Animals will be monitored for signs of distress or weight loss, and will immediately be removed from the study if these are detected. For experiments in which conscious animals are necessary, we carefully monitor them throughout all sessions.

We continually seek to refine our experimental approaches to keep up with the state of the art in our research field. We have been early adopters of new methods (such as chemogenetics) that have allowed improved, more powerful experimental designs using within animal comparisons and reduced need for implants. We have also introduced high density neuronal recording systems that has increased data yields and reduced the numbers of animals needed for experiment. By monitoring spontaneous rather than evoked behaviour and using automated assessments based on video recording we have sought to both reduce stress and make behaviours more naturalistic. During the course of this project licence, we expect this process of refinement to be continually embedded in our thinking about experimental design and methodological improvement. We will consult with NVS/NACWO throughout the duration of the project and will regularly review the options for refinement of our procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidance to improve our reporting of research using animals, maximising information published and minimising unnecessary studies.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are members of academic societies that have a clear focus on 3Rs and experimental approaches for the study of pain and physiology. We attend and present at 3Rs events along and are informed by the regular circulation of information from the NC3R's and keep abreast of developments at www.nc3rs.org.uk.

69. Immunotherapy of amyloidosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Amyloidosis, antibodies, immunotherapy, type 2 diabetes, Alzheimer's disease

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Amyloid is an abnormal material, derived from the body’s own substances, that accumulates within the body causing a number of different diseases. The body removes amyloid very slowly, if at all. We have previously shown that amyloid removal can be accelerated by administration of antibodies directed against it. Antibodies are the proteins the body produces to protect against bacteria and viruses by clearing them from the blood and tissues. We have designed novel antibodies intended to clear away amyloid from the tissues and thereby benefit patients suffering from diseases associated with amyloid. This project aims to demonstrate this desirable activity in vivo, to identify the most effective of such antibodies and to characterise their mode of action so that they can be developed into new medicines for treatment of patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Amyloidosis is a relatively rare but inevitably fatal disease, caused directly by amyloid deposition in the tissues. It kills about one person per 1,500 who die in developed countries. In contrast, type 2 diabetes and Alzheimer's disease, which are also associated with amyloid deposits, are extremely common and carry immense medical, social and economic costs. Treatments for amyloidosis are limited, are often dangerously toxic and do not exist for some forms of the disease. Treatment of type 2 diabetes is challenging and often poorly effective. There is no disease modifying treatment for Alzheimer's disease. These conditions are thus all major unmet medical needs. Our invention of a new approach to treatment, using antibodies, offers a unique opportunity to stop amyloid-related diseases from getting worse, thereby reducing illness and saving lives.

What outputs do you think you will see at the end of this project?

The primary output will be identification of new antibodies that are most effective foramyloid removal and the characterisation of their mode of action in vivo. This new scientific information will be widely shared by publication in high impact scientific journals and presentation at national and international scientific meetings.

The secondary, but vitally important, output of the project will be the information essential for ongoing development of one or more selected antibodies towards clinical testing in patients. The cost of successful development of a new medicine from its invention to its registration as a licensed medicine is around £500 million to £1 billion. Securing this scale of investment demands very compelling evidence of the target validity and the safety and efficacy of the proposed intervention. The results of the present study will comprise the crucial initial evidence.

Who or what will benefit from these outputs, and how?

In the first months the outputs will be new scientific information on the capacity of unique new antibodies to promote regression of amyloid deposits in vivo. If positive results are rapidly obtained, the mechanism of the treatment effect will be characterized within one or two years. This knowledge will be widely shared in the scientific community. Meanwhile the results will enable ongoing development of the optimal antibodies into potential medicines for clinical testing in patients with the fatal, largely untreatable, amyloid-related diseases. If the programme is successful, the effective disease modifying new treatment that will emerge, potentially within 2-5 years after the end of the project, will reduce illness and suffering, retain and restore quality of life, and prolong life in some of the most intractable diseases. Successful modification of the prevalent diseases, type 2 diabetes and Alzheimer's disease, will also carry massive social and economic benefits for humanity and society in general.

How will you look to maximise the outputs of this work?

All positive new knowledge obtained in the project will be widely disseminated as soon as possible via the scientific and medical literature, national and international meetings.

Positive results will also support the resourcing essential for medicine development. The ultimate purpose of the study is to create new medicines to reduce morbidity and mortality in both rare and prevalent diseases that currently lack effective disease modifying treatments and are therefore major unmet medical needs.

Very importantly, we will also publish all negative outcomes. If the newly created antibodies either do not promote amyloid regression in vivo and/or are not safe and well tolerated, we will ensure that this information is widely and readily available when the programme is abandoned.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Amyloidosis is exclusively a disease of adults. It does not occur before adulthood in humans or in any other species. Adult mice, with mature, fully developed systems, are therefore appropriate for this study and mice are the only experimental animals in which forms of systemic amyloidosis can be induced that resemble the human disease closely enough to be informative. Furthermore, the mouse models have been extensively refined to minimise harm to the animals and reduce the numbers used.

Untreated human systemic amyloidosis causes progressive organ failure and is inevitably fatal. Even with the best available treatments, up to 25% of human patients with systemic amyloidosis still die within 6 months of diagnosis. However, in contrast, the amount of amyloid formed in the refined mouse models is very limited and the mice develop no symptoms nor any clinical or biochemical signs of organ damage.

Past use of the mouse systemic amyloid A type amyloidosis model has directly enabled major improvements in the diagnosis and treatment of amyloidosis worldwide. Our unprecedented new transgenic mouse model of systemic human transthyretin (TTR) amyloidosis (ATTR) incisively extends our ability to develop improved treatment for what has lately been recognised as probably the most prevalent form of human systemic amyloidosis.

Median survival in human systemic amyloidosis in 1979 was 15-18 months from diagnosis, whereas it is now almost 10 years in the best specialist centres. Nevertheless, with about one in four patients with systemic amyloidosis still dying within 6 months of diagnosis, new treatment to save these lives, such as this project aims to develop, is urgently required. The present mouse models also provide unique proof of concept information directly relevant to potential applications of the new treatment in all the different forms of human amyloid related diseases.

Typically, what will be done to an animal used in your project?

For induction of amyloid A type amyloidosis, each animal will receive a single intravenous (IV) injection of so-called amyloid enhancing factor, followed by 10 daily subcutaneous (SC) injections of 10% casein in saline, spread over two weeks. All the mice have developed systemic amyloid A type amyloidosis by the end of the course of casein injections. Control mice receive no further treatment. The experimental mice receive one intraperitoneal (IP) injection of the putative therapeutic antibody. All animals are humanely killed between 1 and 28 days after the antibody dose. In some protocols the mice also receive either one IP injection of an innocuous isolated pure protein in saline solution, or three innocuous IP injections of liposomal clodronate before being killed.

All injections will be administered to conscious animals. Amyloid enhancing factor is an extract of amyloidotic mouse spleen, containing amyloid fibrils which serve to trigger new amyloid formation in vivo and thus greatly accelerate and enhance systemic amyloid deposition in the recipient mice. This enabled powerful refinement of the mouse model, notably reducing the number of animals needed and the duration of each study.

For induction of transgenic human ATTR amyloidosis, each transgenic mouse expressing the amyloidogenic S52P human TTR variant on the mouse TTR and α2 antiplasmin gene deletion background, will receive one IV injection of human ATTR fibrils to seed systemic deposition of human ATTR amyloid deposits. All mice develop the desired amyloid deposits, especially in the heart, by 3 months after seeding. At this point, some test mice will receive isolated pure human serum amyloid P component (SAP) by IP injection, an innocuous normal human plasma protein, in saline solution, followed 24 hours later by polyclonal IgG antibodies against human SAP by IP injection. Other test mice will receive one of the new putative therapeutic antibodies by IP injection. Control mice receive no treatment after the seeding injection. All mice are killed 14-28 days after the antibody injection.

What are the expected impacts and/or adverse effects for the animals during your project?

The subcutaneous casein injections produce local chronic inflammation with slight thickening of the skin but no lasting effects and no related unusual behaviours. None of the other materials being injected cause any clinically appreciable adverse effects and we

have never observed distress beyond that associated with restraint and the transient discomfort of the injection itself. There is no weight loss or abnormal behaviour.

The amount of amyloid that will be deposited in the tissues is consistently below the threshold of organ damage and clinical disease and does not affect the natural life span. Amyloid A type amyloid is principally in the spleen and liver with variable small amounts in the kidneys but there is no impairment of liver or kidney function and splenic amyloidosis is clinically silent. The transgenic human ATTR amyloid deposits similarly do not cause clinical illness. However, the amyloid load in both models is sufficient to enable its reduction by our treatment to be sensitively and precisely detected. All mice will be humanely killed promptly at the end of each of protocol.

Antibody treatment was invented and developed specifically to safely remove amyloid deposits from the tissues and this is exactly what happens. Extensive studies in the mouse model happily showed no adverse effects at all. Furthermore, translation of the invention into a treatment for human patients with systemic amyloidosis was safe and well tolerated and produced unprecedented, clinically beneficial, removal of amyloid. The new and completely different antibodies that will be tested in the present project are designed to be inherently even safer than those investigated previously as well as more effective and with a broader range of action. No adverse effects are expected but if they occur, the antibodies responsible will not be tested further.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 100% mild severity category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Amyloidosis is a very complex disease in which the body's own proteins form abnormal fibres, called amyloid fibrils, that accumulate and persist in the space between and around the cells of tissues and organs. The body has very efficient mechanisms that normally remove all dead cells and protein debris from the tissues but amyloid is not adequately cleared in this way. Amyloid deposition disrupts the normal structure of the tissues, damaging their functions and causing disease. Reducing the rate of amyloid accumulation

can arrest disease progression and sometimes restore damaged organ function, providing clinical benefit. However, most of the available treatments for amyloidosis are invasive, potentially lethally toxic and/or of limited effectiveness. There are no treatments at all for some forms of amyloidosis. There is therefore an urgent need for new treatments that swiftly and safely remove amyloid from the tissues. The present project aims to develop such treatment but its potential efficacy in living patients cannot be demonstrated by in vitro studies.

The critical steps required for effective amyloid removal are as follows. Firstly, the antibodies introduced into the circulation must reach the amyloid deposits in the tissues and bind specifically to them. Secondly, the bound antibodies must efficiently activate an extremely complex system of different interacting blood proteins, called complement, that the body uses to remove unwanted materials from the tissues. Thirdly, complement activation must attract a specific subset of cells, called macrophages, that patrol the tissues to conduct waste disposal, so that they accumulate around the deposits. Fourthly, these macrophages must then fuse together to form huge giant cells. These are the only cells in the body capable of surrounding, engulfing and destroying the massive amyloid deposits of systemic amyloidosis.

This extremely complex interlocking series of processes, deliberately set in motion by the antibodies, cannot be replicated in vitro. In vivo testing is essential to establish that the antibodies can reach and bind to amyloid deposits in the living mouse. Complement is an extremely complex, highly dynamic system of at least 21 different interacting proteins, the full relevant activities of which cannot be precisely reproduced in vitro. There are many different types of macrophages and, although some can form giant cells in vitro, there is no evidence that these artificially created cells have the same functions as the authentic cells that form in vivo in response to the antibody treatment. The present, newly created antibodies have been designed to be safe and optimally effective in amyloid clearance but the only possible way to demonstrate these properties, and thus to justify their eventual clinical testing in humans, is to perform the planned in vivo studies in mice.

Which non-animal alternatives did you consider for use in this project?

The novel antibodies to be tested have been selected in vitro from a phage display library, without any animal involvement. They have been created by recombinant technology in the desired antibody class known to activate the complement system because that is the proven basis of our treatment invention. They have been expressed by cell culture with no animal involvement. This is the limit of what can be achieved without testing in living animals.

The objective of the present project is to identify one or more of our novel antibodies that may be suitable for development as drugs to remove amyloid deposits from the tissues of patients. Amyloidosis itself and the many, different, interacting processes involved in the mechanism by which our intended treatment works in the intact living body, are extremely

complex. They cannot be usefully represented by cell or tissue culture studies. Thus no non-animal alternatives for this project exist.

Why were they not suitable?

There is no non-animal alternative for demonstrating that, after introduction of the antibodies into the circulation, they will bind to amyloid deposits in the tissues, activate complement and attract macrophages to the site, nor for investigating the antibody dosage required to produce these effects in the whole living animal. There is no non-animal alternative for demonstrating that the attracted macrophages will fuse into giant cells with the unique ability to surround, engulf and destroy the amyloid. There is no non-animal alternative for demonstrating that these desirable processes can be activated safely in the intact living animal. Robust relevant evidence of this safety and efficacy is essential to justify the development of appropriate antibodies towards clinical testing in human patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The animal numbers are based on extensive published experience with the present disease model. All mice, treated as described here, develop amyloidosis. The numbers of 10-12 mice in each test and control group are the minimum necessary to yield statistically significant differences when the antibody treatment is at least 50% effective in removing amyloid. The PREPARE guidelines and the NC3Rs (design assistant) website guidance will be followed when possible and the studies will be appropriately blinded and randomised.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our mouse amyloidosis models very closely resemble different forms of the human disease. Indeed, as animal models of a complex human disease, they are almost uniquely similar to their human counterpart. Induction of amyloid A type amyloidosis, in mice as in humans, depends on sustained production by the liver of greatly increased amounts of the circulating blood protein, serum amyloid A protein (SAA). This increased production is an automatic response to acute and chronic inflammation. In the mouse, the least severe effective inflammation is elicited by a series of SC injections of casein.

In contrast with variable and unreliable induction of experimental mouse amyloidosis by other groups using 6 weeks of daily casein injections, we have refined the method by using amyloid enhancing factor, an extract of amyloidotic tissue containing preformed amyloid fibrils, to accelerate amyloid deposition. In addition, we have used our novel non-invasive whole body imaging procedure to confirm the presence and amount of amyloid without subjecting mice to unnecessary procedures. We have thereby greatly reduced the number of injections, increased the efficiency of amyloid induction to 100% and thus substantially reduced mouse numbers in all experiments.

Our new mouse model of human ATTR amyloidosis, similarly closely replicates key aspects of human hereditary systemic ATTR amyloid caused by amyloidogenic mutations in the human TTR gene. In particular, the presence of ATTR amyloid deposits in the myocardium is critically important and our design, including both overexpression of the transgenic amyloidogenic TTR variant and knock down of α2 anti-plasmin, delivered 100% efficiency in speedier production of larger amounts of amyloid. This contrasts with all other mouse models claimed to be representative of human ATTR amyloidosis and substantially reduces the number of animals to be used in this project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. All mice used for study of amyloid A type amyloidosis will be purchased from the leading licensed suppliers(s) of experimental mice routinely used by the facility where the project will be conducted. We are relying on their conduct of responsibly efficient breeding. The minimal necessary numbers of transgenic mice with ATTR type amyloidosis, sufficient only for robust experimental results, will be efficiently bred in house.

2. The experimental protocols, including the numbers of animals, were optimised in our previously published work and this successful past experience provides a robust basis that makes pilot studies redundant. There is no evidence to suggest that the isolated, pure, microbiologically clean antibody preparations which we will test will not be safely tolerated. The protocols already specify the minimum numbers required to provide statistically and biologically significant results. Pilot studies would therefore use more animals but could not contribute new or useful information.

3. Computer modelling has no relevance to our programme at this stage. We require robust, reproducible evidence that our newly created antibodies have a beneficial effect in vivo in the one available animal model. The optimised protocols to be used in this model were developed and used under our previous licences. They have been extensively reported and received universally favourable objective recognition and acclaim, as well as academic and commercial financial support for being both appropriate and effective.

4. Unfortunately, there is no scope for sharing tissue between different experiments within the programme in order to reduce animal numbers. Each individual experiment absolutely requires its own control and experimental groups. Our input on the details of the

experiments enabled our statistical adviser to perform robust power calculations to confirm the minimum number of mice in each group needed to deliver statistically and biologically significant results.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The inbred wild type C57BL/6 strain of mice we shall use are ideal for the present project because we have highly refined, very well developed and thoroughly characterised models of systemic amyloidosis in them, which very closely resemble human disease. The induction of amyloid A type amyloidosis requires a single intravenous injection and 10 subcutaneous injections, all of which are of mild severity. There is no appreciable untoward effect other than the extremely rare, less than 1 in 100, occurrence of skin ulceration. If this occurs, the affected animal is promptly humanely killed. The induction of transgenic human ATTR amyloidosis requires just a single IV injection in the genetically modified mice.

We have seen no deaths in work conducted under previous Project Licences. We have very extensive experience of administration of a wide range of different antibodies to mice, with no deaths or appreciable untoward effects, and we therefore do not anticipate any adverse effects with the present antibodies. All mice will be killed promptly by a humane method at the end of each experiment, not more than 60 days after the first injection has been administered, to provide the tissues required to detect the effect of treatment.

Why can’t you use animals that are less sentient?

The mouse amyloidosis models are the only logistically tractable models that exist in mammals. There are no relevant models of amyloidosis in other types of animals. The unavoidable time course of several weeks prohibits the use of anything other than unrestricted conscious animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Monitoring for any adverse effects, including pain, will be the highest priority, although the procedures do not cause more than minimal discomfort, for which pain relief medication has not previously been required. The use of anaesthetics for some subcutaneous

injections has been added recently. This will make the injections less stressful for the staff involved and also for the animals, especially towards the end of the second week of amyloid A type amyloidosis induction.

Two of the substance types to be administered by intraperitoneal (IP) injection, namely human serum amyloid P component (SAP) and the various anti-amyloid antibodies, cannot be formulated at sufficiently high concentration for the required doses to be administered in single small volumes. One or two larger volume injections, up to a maximum of 50 ml/kg, will therefore be used, but only one injection per day. This avoids giving multiple smaller volume injections on several consecutive days which is much more stressful for the animals. IP injections of up to 50 ml/kg are safe and well tolerated in adult mice (AA). The Table below reports our most recent past experience of 50 ml/kg IP injection of materials of precisely the same type that we shall now be testing using this route. In all these experiments, all the mice that received 50 ml/kg IP injections were closely and repeatedly observed by the present licence holder who gave the injections and by the technical staff of the UCL Royal Free Campus BSU facility. No behaviours suggestive of, or consistent with, pain, discomfort or distress were ever observed. No mice died. In a small subset of experiments in which plasma biochemistry was monitored, no abnormalities were detected despite the mice having substantial renal and hepatic amyloidosis. We are therefore confident that the injected materials themselves are harmless.

Furthermore, the absence of adverse effects or any deaths among nearly two thousand animals receiving one or two injections of 50 ml/kg ml each is strong evidence that these volumes are safe and well tolerated. For the avoidance of doubt, none of the other materials to be administered to the mice by IP injection or any other route will involve volumes exceeding the Campus guidelines.

Furthermore, in addition to the usual careful observations, all mice receiving IP injections greater than 20 ml/kg will be weighed at least 2 hours afterwards, to monitor the clearance rate of the injected volume.

Finally, IP injection volumes greater than 20 ml/kg and up to 50 ml/kg will only be needed in (a) protocol 2, which will probably only be deployed 2 - 3 times; (b) in protocols 2 and 11 involving human serum amyloid P component (SAP), which will likely be deployed 2 - 5 times; (c) in all other protocols only if protocol 4, the dose response study, demonstrates that optimal efficacy requires antibody doses greater than 4 mg/mouse. If such high doses are needed for measurable efficacy, the project might be terminated. If acceptable antibody activity is detected, however, the total number of experiments to be done under all the protocols will probably be between 13 - 25.

Intraperitoneal injections, one or two within 4 days, each 1 ml, in ~20g mice with systemic AA amyloidosis					
	single injection volume/mouse	concentration	number of mice injected	distress or other observed adverse effects	deaths
Human SAP	1 ml	10 mg/ml	800	0	0
Polyclonal sheep IgG	1 ml	50 mg/ml	350	0	0
Monoclonal IgG	1 ml	up to 5 mg/ml	620	0	0
		Total	1770	0	0

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The guidelines issued by LASA and NC3Rs, encompassed by Babraham Campus guidelines, will be strictly followed with the single exception regarding the maximum IP volume required for just the two types of administered substances specified and justified in the previous section above.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

NC3Rs resources (website, publications, etc.). In addition, we scrupulously scan the literature in the amyloidosis field, with automatic notification of all relevant publications as well as citations of our published work by others. This is a reliable way of detecting any advances in the 3Rs that may be relevant to the present programme.

70. Strategies to improve donor organ function in a porcine model

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

No answer provided

Animal types	Life stages
Pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to treat organs from donors in such a way as to improve their suitability for transplantation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Because of the increasing clinical need, it is necessary for transplant clinicians to consider the use of increasingly sub-optimal donor organs. Although transplantation is highly effective (more than 90% of liver transplant recipient are alive 12 months after surgery), the discrepancy between the supply of donor organs and the need for them results in patients dying on the waiting list. A patient is now more likely to die on the waiting list for a liver transplant than in the first post-operative year. However less than two thirds of deceased donors in the UK result in a liver transplant; this is because many organs are deemed to be

high risk (more likely to fail post-operatively) due to various factors including: fat deposition; hypoxic injury; older age; cardio vascular disease; diabetes.

Improving donor organs suitability for transplantation This will increase the number of organs available for patients on transplant waiting lists without increasing the risks that the organs will fail to function.

What outputs do you think you will see at the end of this project?

During the term of the previous PPL much progress has been made in terms of the refinement of normothermic machine perfusion and, importantly, it's application. In particular the earlier work on liver perfusion has been developed substantially in the context of the liver, and there is now a design for a prototype device which requires evaluation and testing as part of a pre-clinical development programme. Regarding the pancreas, normothermic machine perfusion has proved to be very challenging and we are now studying a number of technical variations based, particularly, on different approaches to temperature transitions as well as haemodynamics. This work will require considerable input from animal based studies. Also, for all organ types, the issue of delivery of organ specific therapy is very much the target. This includes drugs, cells and genes to mitigate the ill-effects of organ retrieval and preservation

The underlying purpose of this project is to develop strategies to allow the reliable transplantation of donor organs that are outside current safe limits. Normothermic perfusion is likely to be central to this strategy as a therapeutic modality in its own right, but it also provides a model in which other strategies can be tested in a consistent manner without subjecting large numbers of animals to transplantation. It is likely that more than one effective strategy will be identified and that the effect of these will be additive or synergistic.

The measurable outputs of the study will include (i) publications, (ii) presentations to specialist conferences, (iii) translation of novel organ reconditioning strategies to clinical practice.

Who or what will benefit from these outputs, and how?

The potential benefit of this work is very considerable. At present 10 to 30% of patients on the waiting list die before receiving an organ transplant and many more who would benefit from a transplant are not placed on the waiting list. There is a very large population of potential donors from which organs are not transplanted because the risk that the transplant will fail is too great. If it were possible, first, to repair and quantify injury to the organ sustained before retrieval, second, to minimise the damage that occurs to the organ during preservation and then reduce the impact on reperfusion this will result in more organs for transplantation. In addition assessing the viability of the organ before committing a patient to a transplant, then such 'marginal' donor organs could be used in large numbers and safely.

The data and interpretation that result from this work will be made available to other research and clinical groups around the world. Normothermic perfusion is increasingly seen as a vital development in transplantation to improve the quality and testing of donor organs and strategies developed as part of this project will be adopted elsewhere (as has proved the case in the past).

How will you look to maximise the outputs of this work?
We will publish the results of our studies and present these at national and international conferences. We will work with commercial collaborators to ensure that any benefits of the work are translated into clinical practice as efficiently and rapidly as possible.

Species and numbers of animals expected to be used

- Pigs: 180

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We need to use these animals as they will respond to an intervention in a similar physiological way to humans. It is important that the organs we use are similar in size and function to human organs. The pig is widely regarded as the best pre-clinical model for organ preservation studies, prior to trials in patients.

Typically, what will be done to an animal used in your project?

Each animal will receive an injection as pre-medication which will make it drowsy and remove distress. A general anaesthetic will be given, using appropriate methods. When the animal is fully anaesthetised, a breathing tube will be placed in the airway in order to maintain the anaesthetic safely. Tubes will be laced in an artery and vein the neck in order to monitor the condition of the animal during the anaesthetic and provide fluid. The operation will include a mid-line incision in the abdomen, mobilisation of the relevant internal organs and the insertion of a large tube in the aorta for the purposes of retrieving blood and/or administering cold preservation solution to the organs. The procedure will be terminated by administering a large dose of a drug.

What are the expected impacts and/or adverse effects for the animals during your project?

To avoid distress, animals will be sedated before the anaesthetic. The anaesthetic will be monitored by a fully qualified veterinary surgeon. No animal will be recovered from the procedure.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

Non Recovery 100%.
What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals, as they will respond to an intervention in a similar physiological way to humans. We need to use animals to see the response they have to the therapies we use to see whether they would be likely to work in humans.

Which non-animal alternatives did you consider for use in this project?

Cell cultures.

Why were they not suitable?

Whilst suitable for initial hypothesis generation, the behaviours and function of a whole organ cannot be predicted by extrapolation from the behaviour of individual cell lines in isolation. Such data would not be admissible as a justification for proceeding to clinical application

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based this on our experience in recent years regarding the frequency with which our research programme has required studies of this sort and used this on which to base a 5 yearly total.

Wherever possible we will retrieve multiple abdominal organs from the same animal (liver, kidneys, pancreas) . This will reduce the number of animals required to support the necessary studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all pig studies we use the minimum number of animals necessary for the results to be scientifically meaningful. Depending on technical aspects this is approximately 6 pigs per experimental group. We are aiming to identify strategies or treatments that show a clear benefit that do not need large numbers in order to demonstrate significance.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will make the maximum use of the results from every experiment. We will validate findings from the pig model using discarded human organs prior to designing trials in man.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The pig has been chosen for these experiments as it is widely accepted at as the best model for studies that can be related to use in humans. The anatomical and physiological characteristics are similar to those of humans and the pig is known to be more susceptible to preservation damage than the human. The preservation characteristics of the organs and the perfusion methods that are available are substantially different in smaller animals, and so any experiments done in rodents would not be applicable to humans.

Why can't you use animals that are less sentient?

The size requirement is a critical factor in these experiments and the pig is the most suitable model. These experiments will be conducted under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will insure that the sedation and anaesthetic protocols are optimised and consciously seek methods to refine these. We will continuously seek ways of reducing the need for animal studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the published resources atwww.nc3rs.org.uk.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Through routine review and discussion within both the transplant research group and the team at the Biomedical Services.

Regular communication and updates with the team at Biomedical Services.

71. Investigating immune responses in mice

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

antibodies, affinity, diversity, immune modulators

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project proposes to investigate if the immune response in mice can be improved by giving them substances to enhance it. Mice will be immunised with and without enhancement to compare the antibodies they make and show that enhancement leads to better quality antibodies

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue **after the project has finished.**

Why is it important to undertake this work?

Generation of antibodies to make new medicines by immunisation is a well-established process, this project will investigate methods to improve their quality. This knowledge can then be used in the discovery of new antibody treatments and vaccines for patients.

What outputs do you think you will see at the end of this project?

From this project we will see if enhancing the immune response in mice leads to better quality antibodies. This knowledge can then be applied in the discovery of new antibodies to treat patients.

This work will form part of a PhD thesis that will be published and will lead to peer reviewed journal publication(s) to further scientific knowledge in the community of antibody discovery.

Who or what will benefit from these outputs, and how?

If successful, the main benefit is that it will provide a method to generate pools of antibodies which have more diverse properties. This can then be used in therapeutic antibody discovery and allow project teams to select better antibodies with greater binding and specificity attributes to be developed in to medicines. If this then became a standardised technique it would lead to a reduction in the number of animals required for antibody discovery as multiple immunisation groups would be avoided.

The potential future implications of this project would not be limited to therapeutic antibody discovery but could also be applied to vaccine development for pathogens such as Covid, HIV or influenza.

How will you look to maximise the outputs of this work?

If successful, this work would be used in therapeutic antibody discovery and allow project teams have a better panel of antibodies to choose from in developing new treatments for patients.

Species and numbers of animals expected to be used

- Mice: 120

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of Life stages.

We are using transgenic adult mice that have stably genetically altered to use human antibody genes and therefore make human antibodies that can be used to treat disease in humans. As we are aiming to develop new improved methods of immunisation to make better antibodies for treating human disease these mouse strain is the best choice.

Typically, what will be done to an animal used in your project?

Immunisation involves giving a substance to an animal usually by injection under the skin (subcutaneous). The mice will only experience a very short and slight discomfort. Anaesthesia may be used for restraint purposes.

Mice may be given immune modulator treatment either post immunisation by subcutaneous injection or throughout the entire study by injection in to the intraperitoneal cavity.

Once the immunised mice have generated suitable levels of antibodies, as confirmed by taking blood samples and testing in an assay, they will be humanely killed and their immune tissues (lymph nodes, spleens, etc) will be removed to enable us to isolate the antibody producing cells.

At the end of each immunisation study, any remaining mice will be humanely killed by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Around 90% of immunisations will cause no more than transient mild pain to the mice. A small number of mice can exhibit some general signs of being unwell: for example, they may show: weight loss, show behaviour which is quieter than usual (subdued), changes in their posture, changes in their coat, shaking for a short time, runny nose or make more noise than usual. Mice are expected to recover over a 48 hour period.

Rarely (<2%) adverse effects that can occur are inflammation at the site of immunisation that can cause swelling, scab formation or bleeding.

After the mice have been immunised there is a approx. 1% chance that a mouse may suffer an extreme allergic reaction (called ‘anaphylactic shock’) where mice will develop difficulty breathing and/or low blood pressure that could lead to suffering. This is nearly always associated with final boosting. When this happens, the mice are immediately humanely killed to prevent any suffering.

The immune modulators are not expected to have any adverse effects of their own, and any adverse effects observed would be mild, transient and associated with the dosing route.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is expected that 90% of mice will be in the mild severity

It is possible that up to 10% of mice may go in to moderate severity

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This PhD project is to investigate the effect of immune modulators on the immunisation process in mice to see if they can improve the outcome in terms of making better antibodies. There are no non-animal alternatives that can mimic the immune system in how it generates and develops an immune response. Therefore, it is not possible to investigate the effects of immune modulators on this process without the use of animals.

Which non-animal alternatives did you consider for use in this project?

We do have access to non-animal antibody discovery platforms have been considered.

Why were they not suitable?

These are not suitable as they cannot mimic how mice generate and develop an immune response. Non-animal methods are not capable of mimicking the process of antibody generation following immunisation and the subsequent maturing of that response. As such it is also impossible to investigate the effect of immune modulators on this process using non-animal antibody discovery platforms.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any.

These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is based on a typical study design, that has been extensively used over many years, and animal numbers that are required to give significance to the output.

Added to this is additional mice in case it is necessary to repeat all of the studies should there be complications in understanding the outcomes or unexpected observations that need to be investigated further.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Design of experiment involving consultation/review by a statistician is used by default as part of the robust study design initiative when planning immunisation groups.

A statistician will also be part of the review panel for the in-vivo work to ensure that statistically significant observations can be made and compared for each animal group used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our historical immunisation data with this transgenic mouse to calculate how many mice have responded to immunisation in the past and therefore predict how many will be needed for these studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using our most optimal mouse immunisation model for generating a robust immune response to immunisation in this project. As part of this work we will be comparing the immunisation outcome of mice that follow the standard immunisation procedure and compare this to the outcome of standard immunisation combined with immune modulator treatment.

Mice will be handled by non-aversive methods such as tunnel handling and environmental enrichment will be added to their cages.

These immunisation procedures will be of the shortest duration with the minimum number of injections (hence minimising any pain, suffering, distress and lasting harm) required to generate the data to answer our experimental questions. All the reagents used will have been certified as fit for purpose prior to animal work starting.

Support for animals will be provided as necessary, e.g. heat mats post immunisation, enriched softened food and pain relief.

Once we have the data required no further immunisations to produce additional data will be initiated.

Where animals have to undergo repeated injections as part of the protocol, injections sites will be changed between each injection. Also the sites of injection will be monitored daily for inflammation and/or irritation to allow immediate intervention if required.

Why can't you use animals that are less sentient?

Less sentient species do not have a functioning immune system equivalent to that of mice making them unsuitable/unusable for this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Prior to immunisation, all immunogens and immune modulators are stringently checked for contaminants and sterility to ensure there is no potential for causing illness during the immunisation. Furthermore, any immune modulator used is thoroughly researched for any potential adverse effects that could occur prior to being used.

Our immunisations have been refined to minimise any distress because of the immunisation process. Our examples of this are:

- Minimising pain during the immunisation by using fine gauge needles and using anaesthesia when multiple injections are required.
- When using substances that improve the immunisation process, we make sure these do not cause any additional suffering or distress on the mice by thoroughly researching the immune modulator. Any identified adverse effect would be discussed with the NACWO and NVS.
- We provide supportive care (e.g. provision of soft diet and warming) for any mice that are affected by immunisation to reduce any pain and help them recover as quickly as possible

If any of our mice reach predetermined humane endpoints during the immunisation protocol, they are humanely killed to prevent any further suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The published principles and philosophies behind the PREPARE (2018) and original ARRIVE (2010) guidelines have been incorporated into the sponsoring company's internal project planning standards of care and standard operating procedures. All work carried out under authority of this licence will undergo assessment of the study design during planning stages as part of a peer review process that is based on those guidelines, and will include statistical consultation. Facilities and processes are audited by independent bodies such as AAALAC International which has published guidelines and procedures to ensure work is carried out to high ethical and humane standards. The following published documents will advise on experimental design, animal welfare and husbandry during the life cycle of this licence:

i. Kilkenny C et al (2010). Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. PLoS Biol 8(6).
ii. Smith A et al (2018). PREPARE: guidelines for planning animal research and testing. Lab Anim; 52(2):135-141.
iii. Percie du Sert N et al. The ARRIVE guidelines 2019: updated guidelines for reporting animal research. BioRxiv. 2019: 703181.
iv. NC3R's - Responsibility in the use of animals in bioscience research: expectations of the major research council and charitable funding bodies (2019).
v. Guidance on the operation of the Animals (Scientific Procedures) Act 1986. (Home Office 2014).
vi. LASA - Guiding principles on good practice for animal welfare and ethical review bodies. (2015)

vii. Prescott MJ, Lidster K. Improving the quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156, (2017).

viii. Review of harm-benefit analysis in the use of animals in research. Report of the Animals in Science Committee Harm-Benefit Analysis Sub-Group chaired by Professor Gail Davies (Nov 2017).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We routinely receive 3R's updates within the company that are sent to everyone involved in animal experimentation from the NIO.

Our AWERB meetings also highlight any 3R's news to PPL holders. Any that a relevant to this project work will be considered and implemented.

We also receive email updates from EARA.

Our research department uses non-animal alternatives for antibody discovery where possible to deliver therapeutic antibodies.

72. Generating new models of human cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, models, genetics, therapy, oncogenes

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To generate and preserve genetically altered mouse strains in collaboration with other scientists working in cancer biology and their collaborators.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer directly affects 1 in 2 people in their lifetime and whilst great strides have been made in detection and treatment there is still much to be learned. For the past 25 years genetically altered animal (GAA) models, particularly mice, have been instrumental in cancer gene discovery, validation and characterisation, and as pre-clinical models.

What outputs do you think you will see at the end of this project?

New Information: This project will deepen our understanding of the underlying causes of cancer in general and of specific types of cancer which currently have a poor prognosis such as pancreatic cancer. Knowledge of the genetic causes will dramatically improve our ability to diagnose, treat and prevent cancer which affects one in two of the UK population.

Publications: Work arising from these studies will be published in scientific journals and presented at national and international meetings to disseminate knowledge (to scientists and clinicians). We will also publicise our results to the public at open evenings, social media, and on our website.

Products: The overall goal of this project is the identification of drug targets which might be used as a therapeutic for the treatment of cancers. This work will also lead to the development of new more accurate and more refined models of human cancers which may be widely used by collaborators.

Sperm or embryos from mouse strains generated in the course of this project will be frozen and archived. These will be made available to other scientists worldwide on request.

Who or what will benefit from these outputs, and how?

This work is aimed directly at individuals with cancer, who will benefit firstly from a better understanding of how this disorder affects them. This should allow a better management of the clinical consequences of condition in affected individuals. The knowledge gained will also be of interest to the scientific community (short term) by expanding knowledge of different cancer types. By the end of this project this work may allow us to develop new therapeutic approaches (long term) helping patients who are suffering with cancer.

How will you look to maximise the outputs of this work?

We will collaborate with a number of different labs both locally and further afield. This will help ensure that the models we generate are used as effectively as possible and that the consequences of the genetic changes will be widely understood in different tissue and cancer types. Our mouse models will be preserved as part of a frozen archive. These new mouse stains will be made readily available to other researchers in the field to study and develop treatments for cancers. In addition, our work will be published in scientific journals and we will attend relevant scientific meetings to discuss and disseminate our findings with other researchers.

Species and numbers of animals expected to be used

- Mice: 34000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Cancer, a major health issue worldwide, is a multistep disease resulting from a series of genetic mutations in genes referred to as oncogenes and tumour suppressors. Understanding how these genetic lesions change the normal cell to a cancerous one is vital if we are to prevent and treat cancer. Only in the context of the complete living animal can we fully understand how cancers develop, invade and spread to other organs. Using genetically altered animal (GAA) models with the same genetic mutations identified in the human disease (so called ‘patient-like’ animal models) we can investigate the biological consequences of these lesions in cancer progression and identify those genetic events and signalling pathways which work together to drive invasion and metastasis. Such information will enable us to design new and targeted therapeutic approaches.

Typically, what will be done to an animal used in your project?

Some animals will undergo hormone injections. Other animals will undergo surgery for vasectomy or embryo implantation.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of new GAA strains we develop are unlikely to display any adverse effect, as only a single gene copy will be affected. Some animals will undergo hormone injection, which is expected to result in no more than transient discomfort and no lasting harm. Some other animals will undergo surgery for embryo implantation; these animals will experience some degree of pain/discomfort will occur as a result of surgery, but this will be minimised by the administration of pain relief medication.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the animals on this project will have a subthreshold severity meaning they will show no harm. Some (<25%) will be mild severity. A smaller number (<10%) will be moderate severity.

What will happen to animals at the end of this project?

Killed

- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although many aspects of cancer research can be conducted using cell lines the development and treatment of cancer can only be properly assessed within the context of the whole animal.

Which non-animal alternatives did you consider for use in this project?

Our mouse experiments are an extension of robust laboratory investigations from work on cultured cells and only progress using mice when sufficient rationale is obtained based on cell culture, including embryonic stem cells and where possible colonies of cells resembling mouse tissues. Where possible we use cell lines derived from human tumours (including 3D organotypic lines).

Why were they not suitable?

Studies using isolated cultured cells cannot totally model the complexities of cancer development in the living organism. It is well recognised that immune and tissue environment play an important role in disease progression where these cannot be fully reproduced in the test tube. For example, drugs which are effective in cultured cells may not be as effective in the whole organism, if they can't reach the tumour or are chemically altered by body cells. So it is always important to analyse promising agents in the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are estimated based on the numbers used during the course of the last project licence. These techniques are all standard techniques allowing the generation of new genetically altered mouse strains.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Although these are all standard protocols, we ensure by monitoring the literature and attending relevant meetings that the methods we use are as close to the state-of-the-art as possible. All reagents we use in mice are firstly tested in the lab to ensure that these work well before animal use. For embryo production we use a special mix of hormones which allows use to generate double the amount of embryos produced by donor mice, halving the number of mice used in these procedures.

When generating a new GAA strain, genetic background will be carefully considered to minimize the number of subsequent breeding steps required to produce cohort mice. Breeding will be optimised, wherever possible, to produce only the genotype required e.g. homozygous breeding pairs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible, unnecessary generation of new GAA strains will be avoided by searching external databases, examples of which are listed below: International Knockout Mouse Consortium (http://www.knockoutmouse.org/); NC3R's Minimising the use of GA mice (https://www.nc3rs.org.uk/minimising-use-ga-mice); Mouse locator (Locator@cancer.org.uk); Jackson Laboratory (http://www.jax.org/ http://jaxmice.jax.org/index.html); Cre transgenic database: http://www.mshri.on.ca/nagy/Cre-pub.html). In addition to the resources listed above we will also check scientific literature to see if strains are available elsewhere.

Preservation of frozen gametes and embryos to archive lines is carried out to avoid generating additional animals, and the need to maintain colonies by continuous breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This licence uses genetically altered mouse models, which aim to recapitulate the same genetic mutations observed in human cancer. Although the protocols for generation of genetically altered mouse strains are well established, we will continue to review the relevant literature and will implement any refinements that are appropriate.

Why can't you use animals that are less sentient?

The mouse is a mammal and warm-blooded which shares many features of human metabolism not found in other cold-blooded species such as flies and worms. With the ease of manipulating the genetics of the mouse, this makes the mouse the best model organism to understand the genetic changes observed in complex diseases such as cancer. In addition, there has been a huge body of work analysing cancer biology in the mouse, and there are already many strains of mice carrying cancer relevant genetic alterations. These can be used in combination with the new mouse strains we generate to effectively understand cancer biology. Consequently, to ensure that these models are efficiently and effectively used we need to generate these using mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In many cases we use models, which specifically target genetic changes to the cell type of interest during adult development thereby reducing the non-specific off-target effects often observed in complete knockout animals.

A small number of animals will undergo surgical procedures. Surgery is carried out under anaesthetic in a sterile manner with appropriate pain relief. Following surgery animals will be maintained in a warm environment, and carefully monitored until fully recovered and returned to home cage. Normal diet may be supplemented with treats to encourage eating and drinking after anaesthetic.

To minimise suffering all mice on procedure will be monitored daily and humanely killed when exhibiting signs of altered health status or other specified end-point is reached. Our animal unit is proactive in enrichment with for example tissue, fun tunnels and nesting material.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all of our studies we will refer to the Guidelines for the welfare and use of animals in cancer research (Workman et al, 2010) and ensure best working practice. All newly generated strains will be assessed according to the published guidelines for best practice (e.g. Wells et al. 2006).

In addition, we adhere to both the PREPARE guidelines: Planning Research and Experimental Procedures on Animals: Recommendations for Excellence as well as the 'Animal Research: Reporting In Vivo Experiments (ARRIVE) Guidelines'. These are complementary checklists for researchers designed to improve the planning and reporting of animal research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continually review our processes and take advice from the Named Veterinary Surgeons, Home Office Inspectors and the NC3Rs website. Our technical staff are very adept at adopting 3Rs advancements such as non-aversion handling and single-use needles.

73. Signalling in normal and abnormal blood cell function

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer, Blood, Stem cells, Therapy, Platelets

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to identify how blood stem cells receive signals and how this drives the formation of mature blood cells, especially red blood cells and platelets. We are particularly interested in finding out how this signaling is altered in blood diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Blood is one of the most important tissues in the human body. Red blood cells transport oxygen to muscles and organs, white blood cells fight infections and provide immunity and platelets control clotting which prevents excessive blood loss after injury. Blood cell production (called "haematopoiesis") is a remarkably complex process by which blood stem cells divide and mature into functional circulating blood cells. A healthy adult makes and turns over about 4 million new blood cells every second. It is therefore critical that we maintain the correct number of blood cells on a day-to-day basis to make sure our tissues remain oxygenated and our immune system is functional. It is also essential that haematopoiesis responds to certain conditions (e.g. bleeding or infection), by either increasing or decreasing the pools of certain types of blood cells and either increase or decrease certain types of blood cell.

In blood diseases, it is common for these systems to become unbalanced. For example, in blood cancers the number of certain types of blood cell are too high and usually do not function normally and block the production of healthy cells. In other diseases, we don't make enough of a certain blood cell type which means they can't do their job properly which can lead to conditions such as anemia and bleeding. In my lab, we investigate how signaling molecules (called cytokines) interact with blood cells and alter their production or destruction. We have found that this control system can be altered in certain types of cancer and other blood diseases. Our research focuses on determining the mechanisms responsible for altering this control system and developing new agents that can intervene and restore normal haematopoiesis.

What outputs do you think you will see at the end of this project?

We will gain a better understanding of how blood cells develop, determine the long-term effects of chronic conditions, and possibly discover new types of agents that may help treat blood cancers. We will communicate our findings widely to basic scientists and medical researchers by publishing open access peer reviewed papers. We will communicate to the public and to schools via our contacts with local patient groups and outreach programs. If our findings point to a mechanism by which minor chronic conditions can develop into severe, long-term disease, this could lead to researchers undertaking further studies in human patients.

Who or what will benefit from these outputs, and how?

Beneficiaries of this research includes the scientific and research communities, as well as patients with blood disorders. We have identified a number of new pathways and agents furthering our understanding of how blood disorders develop. By using this new knowledge, we hope to find ways to prevent or treat blood diseases. This will allow other researchers, including those that work with patients and patient material, to develop new hypotheses and make further discoveries. Our work may eventually lead to the development of new therapeutics and more effective treatments for certain types of blood cancers.

How will you look to maximise the outputs of this work?

Working collaboratively across the university and with researchers at other universities in the UK and internationally has been an important part of my lab's research strategy. We will present new data at national and international conferences such as the British Society for Haematology and the American Society for Hematology to ensure that it is disseminated throughout the scientific community. To promote reproducible and robust science across the UK and internationally, it is important that unsuccessful, as well as successful, experiments are disseminated. Therefore, we will publish any failed approaches on open access servers such as BioRxiv. Scientific research is largely funded by the public, through taxes or through charities, therefore the public have a right to understand what research is being done and our findings. Communicating science to the public is encouraged in my lab and students an postdocs participate in outreach events. We also host a number of patient support groups so our findings are disseminated to members of the public who may have directly or indirectly been affected by haematological disease.

Species and numbers of animals expected to be used

- Mice: 1150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice have a blood cell production system that is similar to that of humans. This allows us to explore how blood is produced, what goes wrong in blood diseases and how we might be able to stop blood diseases from developing by using new treatments. We use adult mice from ~8-12 weeks of age because their blood cell production system is fully functional from this stage.

Typically, what will be done to an animal used in your project?

The mice in our experiments could be treated in several different ways. For some, we may just take very small amounts of blood (like a pin-prick) and look at the different types and numbers of blood cells they have. These experiments tend to last a few months, as we need to see whether any change in blood cell number or type carries on for a long time, or changes when the mice get older.

Others will be injected with chemicals or proteins that alter the number and type of blood cells they produce. They may be injected a number of times and will have small amounts of blood taken at regular intervals to see what effects (if any) the chemicals or proteins

have on blood cell production. Generally, these experiments last a few weeks, but we might keep the mice longer to see if there are any long term effects or whether blood cell production just goes back to normal.

Some mice may receive a bone marrow transplant. In these cases, mice will be exposed to radiation to kill the cells in the bone marrow that make blood, and then be injected with bone marrow cells from another mouse to so they can start making blood again. Mice are closely monitored during this time as they might get sick after being exposed to radiation. However, we have lots of ways to make sure this doesn't happen. These experiments tend to last up to 6 months or even longer as it can take quite a long time for blood cell production to become normal again. To see how the transplant is working, we will take very small blood samples every 4 weeks and look at the types and numbers of blood cells the mice have.

What are the expected impacts and/or adverse effects for the animals during your project?

For most of our experiments, the mice will only experience mild discomfort for short periods of time (such as blood sampling or injecting chemicals/proteins). Any discomfort will be temporary. It is possible that some of the chemicals or proteins may make the mice feel unwell. Mice that are treated with radiation can become unwell, usually about 1-2 weeks after they have been exposed to radiation. This might cause them to temporarily lose some weight.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild: 650 adult mice
- Moderate: 500 adult mice

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We make trillions of new blood cells every day. This is an incredibly complex process that takes place predominantly in the bone marrow - a semi-solid tissue found in centre of long

bones (e.g. legs) or in porous areas of bone (e.g. pelvis and vertebrae). It is essential that we maintain the right balance of different blood cell types to allow muscles to receive oxygen (red blood cells), so we clot properly following injury (platelets) and can fight infection (white blood cells). If this process becomes unbalanced, it can lead to diseases of the immune system or even blood cancers. Controlling what type of blood cells are produced and how many, requires a delicate interplay between signals released from other organs (such as kidneys and the liver) and changes in the bone marrow environment. Therefore, it is not possible to accurately recreate blood cell production in the lab, so we are not able to understand how blood diseases develop without using mice. As the blood cell production system is similar between mice and humans, we are able to gain a good understanding of this process using mice.

Which non-animal alternatives did you consider for use in this project?

As blood cell production is so complex, there are no non-animal alternatives for these studies. There are some in vitro (using cells in the lab) assays that allow us to determine how certain agents or diseases effect blood cell development. We will use these where possible and if these assays are appropriate. These are especially useful if we need to determine the effects on human cells.

Why were they not suitable?

The in vitro alternatives fail to provide the complexity of the blood cell production, which relies on the highly regulated coordination between many different tissues (such as the liver, kidneys and spleen) and bone marrow. It is not possible to make an accurate model of this in the lab.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As the number and types of blood cells in circulation is tightly controlled, any significant change is easy to identify. We estimate the number of mice we will need to use for each experiment, and how many mice we need for each group. For example, 1 group will be "control" (no treatment) and the other will receive an "agent" (e.g. chemical or protein). Using data from previous experiments, we can estimate how many mice we will need in each group to identify what would be considered a significant biological effect. For example, the normal platelet count in mice is ~800,000 in each microlitre of blood. If we expect the agent to reduce platelet count, we would want to see a reduction of at least

50% (i.e.~400,000/microlitre). We are then able to calculate how many mice we will need to get a strong and reproducible difference.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use a number of online tools to calculate how many mice we need for each experiment, such as the NC3R's Experimental Design Assistant and programs that help us estimate the number of mice we need (http://powerandsamplesize.com/Calculators). One of the most important factors in estimating exactly how many mice we need to an experiment, and therefore making sure we don't use too many, is using our previous data to understand what effects we can expect to see and how similarly mice respond to certain treatments. It is equally as important to make sure you use enough mice to generate good data. If our groups are too small, differences might not be statistically significant, meaning that we will have to repeat the experiment and use more mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

If we are doing an experiment where we are unsure what the effect on the mice will be, we start with a small number of mice first. We then monitor these mice very closely to see what effects (if any) the treatment or experiment is having on the number/type of blood cells. This allows us to understand if the experiment is working, how big an effect is and whether there are any adverse side effects that we didn't expect. Once we have this information, it allows us to more accurately plan a larger experiment. At the end of an experiment, when the mice are killed, we try to collect as much different tissue as possible. This tissue will be frozen and stored in the lab where it can be used by researchers to look at how our experiments may have altered different organs and tissues in the mice. This prevents us, or other groups, from starting new experiments using more mice to investigate effects on these tissues.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use a number of different types of mice for our studies. Often, we will use "normal" mice (called C57/Bl6, or wild type). These mice are widely used in research as a standard. We also use genetically altered (GA) mice. These mice often lack certain genes, or their

genes are altered in some way so they can be used for research into different types of blood diseases. Although the GA mice we use do have certain blood disorders, it doesn't cause them any pain and they don't suffer or die prematurely compared to wild type mice.

The methods we use have been designed and modified over a number of years to significantly reduce pain or distress to the mice. For example, we don't pick mice up by their tails when taking them out of the cage, as this can cause distress. Instead, we either cup them (let them walk into the palm of our hands) or let them walk into a tube before picking them up. We anesthetize mice before taking blood so they are unconscious during the process, only take blood samples when needed and make sure we change the site where we take a blood sample regularly to prevent the area become sore or inflamed.

For bone marrow transplants, where possible, we are using a GA mouse (called W41) that only needs about 20% of the normal radiation dose, this reduces any adverse effects.

Why can’t you use animals that are less sentient?

We use mice because the way they make blood cell is similar to humans. We need the mice to be at least 8 weeks old because their blood system is then fully developed.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are monitored closely after procedures by weighing them regularly and checking on how many and what types of blood cells they have. Mice that have received radiation prior to bone marrow transplantation are given antibiotics for 2 weeks to reduce the chances of bacterial infection.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the guidelines published by the UK Government on best practice for animals in research (https://www.gov.uk/guidance/research-and-testing-using-animals).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We refer to the guidelines published by the NC3Rs (https://nc3rs.org.uk/3rs-advice-project-licence- applicants-refinement) for direction on general refinement measures and to access resources for specific experiments. I read the monthly NC3Rs newsletter and share them with the researchers in my lab that are involved in animal research. I regularly monitor progress on the annual challenges and have previously applied for research funding from The NC3Rs. Our NACWO also sends users of the animal facility regular NC3Rs updates and organizes visits from members of external organisations (e.g. the NC3Rs, RSPCA (https://norecopa.no/PREPARE)) to speak at our 6-monthly users group meetings. During the previous approved PPL, we implemented new NC3Rs

recommendations by changing our mouse handling methods and using needles only once for all our experiments and we will continue to respond to new advances published by NC3Rs.

74. Targeted tumour therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunotherapy, Oncolytic virus, HOX genes

Animal types	Life stages
Mice	adult, pregnant, neonate
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To assess the ability of anticancer agents, either alone or in combination with other anticancer agents, to treat or prevent tumour growth

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

To assess new targeted treatments for cancer. To provide data to support new clinical trials for cancer patients.

What outputs do you think you will see at the end of this project?

We will use cancer killing viruses which retarget the patient's immune system against the cancer. This form of treatment is called viral immunotherapy. Bladder cancer offers intriguing opportunities for viral immunotherapy. Bladder cancer is the seventh most common cancer in the UK, with over 10,000 new cases annually in the UK. Approximately 70% to 80% of patients with bladder cancer are initially diagnosed with an early form of the disease called non-muscle invasive disease. Superficial, non- muscle invasive bladder cancers (NMIBCs) are managed with surgery followed by chemotherapy and/or immunotherapy. The use of bacteria Bacillus Calmette-Guerin (BCG) as an immunotherapy for bladder cancer and its proven effects of reducing recurrence and progression and improving disease- specific survival have revolutionized the treatment of this malignancy. However, the potential for serious side effects of local and systemic bacterial infection as well as the fact that there is a significant (30%) group of non-responder patients to this treatment highlights the need to develop future immune-based therapies that overcome these problems. Our group has developed a novel viral immunotherapy based on a cancer killing virus called Coxsackievirus A21 (CVA21). We have shown that CVA21 has strong immunotherapeutic properties in experimental bladder cancer models and in patients in a clinical trial. A new license would allow us to study a new range of cancer killing viruses expressing bacterial proteins to better target and create a more potent immune response for this disease and for other cancers.

Prostate cancer is the most common non-cutaneous cancer in men worldwide, with an estimated 1,600,000 cases and 366,000 deaths annually. Despite the high long-term survival in localized prostate cancer, metastatic prostate cancer remains largely incurable even after intensive multimodal therapy. Prostate cancers are generally considered to be 'cold' tumours with minimal T cell infiltrates, lacking a type I IFN signature and chemokines and containing immunosuppressive cells such as myeloid derived suppressor cells. This non-inflamed phenotype is thought to be largely responsible for the disappointing lack of sensitivity of prostate cancer patients to immune checkpoint blockade (ICB) therapy. Whilst cancer immunotherapy with checkpoint blockade (ICB) therapy has revolutionized the treatment of patients with certain malignancies, clinical trials in prostate cancer have shown ICB to have very limited efficacy. The potential benefits of these orthotopic models is to allow our group to study a combination of ICB with other immunotherapies, to increase the potential of ICBs seen in other malignancies.

Oesophageal cancer – of which adenocarcinoma (OAC) is the predominant subtype in the United Kingdom - is a highly aggressive malignancy, ranking sixth among all cancers for mortality, with 5-year survival rates of approximately 15%. Surgically resectable disease is primarily treated with chemo+/- radiotherapy and surgery. Systemic chemotherapy is associated with significant side effects and morbidity. While chemotherapy or chemoradiotherapy may modulate the tumour microenvironment (TME) towards tumour rejection, they are unselective treatments with significant systemic toxicity.

Hence novel approaches to the treatment of OAC are required. Immunotherapy may achieve more precise and potent immunomodulation with delivery of biological agents. Creating novel immunotherapeutic strategies which utilise multiple agents to achieve the greatest effect offers the opportunity for significant advances in the treatment of OAC. Innovation in therapeutics has been hampered by a lack of animal models which replicate the tumour microenvironment with an intact immune system.

Who or what will benefit from these outputs, and how?

We hope in the short term, to obtain knowledge for the wider scientific community, which will be disseminated through scientific papers and international conference's. Data from each protocol can be used to help design drug dosing regimens and minimise/eliminate adverse effects in other protocols within this license. Our group has a sustained and proven track record in development of basic science and clinical translation in the fields of cancer killing viruses and therapies that turn the immune system against cancer. Our group has a collaboration with our local NHS Hospital which has a pharmacy, with a dedicated gene/viral therapy facility and a treatment centre with the infrastructure to conduct and manage early phase trials with such biological agents. Thus, in the long term, data generated from this license may translate into clinical trials, which may lead to novel treatments or combinations of treatments for cancer, specifically, for oesophageal adenocarcinoma, prostate and bladder cancer.

How will you look to maximise the outputs of this work?

We will obtain knowledge for the wider scientific community, which will be disseminated through scientific papers and international conference's.

Species and numbers of animals expected to be used

- Mice: 3890
- Rats: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mouse and rat cancer models represent a mammalian system that is well characterized and available in defined genetic backgrounds, greatly reducing the number of variables that could influence experimental outcomes.

Typically, what will be done to an animal used in your project?

Tumours will be inserted into the animals by injection or surgery. Animals with a confirmed tumour, will be treated with anticancer agents over maximum of 4-6 weeks. Tumour load may be assessed by ultrasound, IVIS or x-ray imaging. In some instances, treatment may lead to the regression of tumours. These animals may be re-challenged with fresh tumour cells to look for long lasting immunity against the specific tumour type. Alternatively, immune cell components, may be depleted by antibody neutralization, to understand which immune cells are important in the regression of the tumour. Animals will be culled according to scientific or humane endpoints.

What are the expected impacts and/or adverse effects for the animals during your project?

In all tumour models we monitor changes in: hunching, an altered demeanour, tumour dimensions and tumour ulceration. To minimise suffering of the animals timely monitoring and careful observation of the mice will ensure that any harms are kept to a minimum and when they occur, dealt with promptly.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In our last licence, using experiment tumour models 97.1% of animals show no signs or symptoms of clinical illness and were culled at scientific endpoints (mild). But 2.2% of animals showed an alter gait, 0.22% showed hunching/low demeanour and 0.33% Tumour ulceration and therefore these animals were culled at Humane end points (moderate).

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

One of the key aspects to our research is to study the role that our novel anti-cancer agents play in modulating the immune system to kill cancer. For this we require a live animal with a fully functioning immune system.

Which non-animal alternatives did you consider for use in this project?

In vitro (test tube experiments)

Each treatment is tested in cell culture for tumour killing properties. This data is then compared against well studied anti-cancer agents such as chemotherapeutic agents (eg Cisplatin) on the same tumour cell line. Also, from tissue culture experiments, we can obtain information on the type of cell death caused by anti-cancer agents and whether proteins that will affect the patient's immune system are produced.

Ex vivo (experiments on patient biopsies)

Ex Vivo testing of anticancer agents using the slice culture model using patients tumours

Our group has developed the tumour slice model which is another way we can screen anti-cancer agents. The model allows us to slice tumours taken directly from human patients and from animal models. Cell lines grown in in vitro culture normally contain a single type of cell which is propagated in a single layer. In contrast tumours are complex and multi-layered structures. The tumour tissue slice cultures are preserved in the native state of the tumour, for further study. Tumours are obtained directly from operating theatres at NHS Hospitals. Tumours are slice using a vibrating blade. Tumour slice cultures are maintained under atmospheric oxygen levels. A tumour can be divided into up to 30 slices which allows multiple drugs and viral doses to be tested at any one time. This system has allowed our group to refine and reduce the number of animals needed for our cancer therapeutic studies by allowing us to look at the novel therapies directly in primary human tumour cells. It can also be used with animal tumour slices.

Why were they not suitable?

Limitations of cell culture (test tube experiments)

1. Cells in tissue culture do not contain morphologic structures seen in the tumour in patients. Cell culture also does not contain the mix of population of cells seen in tumours ie stroma and immune cells.

2. Cell culture assays can only be studied over a number of days whilst animal experiments can give us scientific information over weeks. This limits treatment and response time for studying the anti- cancer agent.

3. The cell culture model does not allow us to examine cancer-immune cell interactions. It is essential for our studies to understand the interaction of the immune system with cancer cells and with anti- cancer agents.

Advantages of slice cultures

1. Tumour killing properties of anti-cancer agents can be studied by staining slices with proliferation markers or staining cell death markers.

2. Induction of proteins that may alter the patient's immune system can be measured Which could suggest whether an anti-cancer agent will prime an immune response.

Limitations of slice cultures

1. Currently tissue slices can only retain tissue morphology in culture for between 96 and 127 hours. This limits treatment and response time for studying cancer agents.

2. The slice culture model does not allow us to examine cancer-immune cell interactions. It is essential for our studies to understand the interaction of the immune system with cancer cells and anti- cancer agents.

In conclusion the slice culture model offers a way of pre-screening anti-cancer agents directly onto patient biopsies. It allows us to quantify tumour killing properties of these agents and measure immune response proteins. Unfortunately, however, this does not replace animal studies completely because it doesn't represent a fully active immune system and therefore, we can't fully study the effect of anti- cancer agents.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

It is difficult to estimate the number of animals that will be used before pre-screening of anti-cancer agents has been carried out in vitro. Protocol-1 is a protocol to standardise tumour growth. Previously in 2018 we used 30 mice to study tumour growth in mice. We also expect to use the same number of mice for this objective in this licence to test tumour growth (up to 30 mice per year and up to 150 mice or rats in total over 5 years). However, overall, we will use approximately 50 mice or rats per year (250 mice in total) because an additional 20 mice or rats per year will be required to grow tumours for use in the tissue slice culture model system described above. Protocol-2 is a rodent cancer heterotopic models. Based on our returns from 2016 341 mice were used to test two different types of oncolytic virus therapy or/ and checkpoint inhibitor and another 24 mice were used in HOX gene therapeutic peptide studies. A total over 5 years of 1825 mice. Similar data was obtained on previous project licence. Therefore, we have estimated up to 2150 animals per five years of the project license would be used in protocol 2 of this licence (2000 mice, 150 rats). Protocol-3 is a treatment and prevention vaccination model. Based on our returns from the last set of experiments under this protocol in 2015 97 animals were used for four oncolytic virus vaccine lysates experiments. Based on these previous studies we therefore estimate for this licence we will use up to 750 mice, and 250 rats per five years of the project license for protocol 3. Oesophageal adenocarcinoma (Protocol 4/5) is a new area of research for our group.

Protocol-4 will produce N= 60 L2-IL-1β (Tg[ED-L2-IL1RN/IL1B) mice that have been bred with wt C57BL/6J mice, for use in protocol 5. To breed this group of animal, we require 9 breeding females, each will have 5 litters. Breeding of these GAA mice is controlled by a breeding plan, devised by NACWO, NVS and members of our lab, to minimise animal wastage (https://www.ncbi.nlm.nih.gov/books/NBK43325/). Detailed calculations are shown in appendix 6.

Protocol-5 will use 60x animals produced on protocol-4 to study effect of anticancer agents on oesophageal adenocarcinoma. Protocol 6 is an intra- bone (metastatic) tumour model, which may need 20x animals to set up. Our current design of experiments will use 15x mice per treatment group with two treatment groups in each experiment. We may carry out this protocol 6x times on the license, therefore in total we may use 200 mice per five years of the project license. Protocol 7 is an Orthotopic Prostate Murine Model. This protocol has the same design of and number of experiments as protocol 6, it also will need similar numbers of animals for its set up, therefore we may use 200 mice per five years of the project license. Protocol 8 is an orthotopic bladder tumour model. In the last year this protocol (Previous Project Licence) was carried out we used 97 rats (485 rats/5years) in this procedure.

Therefore, we estimate we will use up to 500 rats or mice (per five years of the project license. Protocol 9 It is difficult to estimate the number of animals that will be used before pre-screening of anti-cancer agents has been carried out in vitro. Group sizes will depend on, which protocol the animals enter after protocol 9. We expect to use 50 mice/10 rats per year and therefore 250 mice/50 rats throughout the license.

As described above, our group has developed in vitro and ex vivo pre-screening procedure for anti- cancer agents, to limit the use of animals used in our studies. If an anticancer agent does not show the desired efficacy in vitro then in vivo experiments will NOT be carried out. It is difficult to estimate the number of animals that will be used before pre-screening of anti-cancer agents has been carried out in vitro, but based on our previous licences we estimate up to 4490 animals will be used over the five year period of the project license. Due to the broad variety of projects being currently investigated in this laboratory the numbers and protocols have been stipulated to cover all eventualities now and in the future. In practice, the actual numbers of animals in use at the moment is below this estimate. It is our intention to continuously reassess in vivo projects and refine them.

The design of the individual experiments will ensure that the animals used are both necessary and sufficient; more animals than necessary would lead to an unethical loss of life with no additional gain in information, whilst insufficient numbers of animals would mean that the animals used are wasted because the scientific question cannot be answered using that data.

Because the tumour models / treatments to be tested are not confirmed before pre-screening of anti- cancer agents has been carried out in vitro, it is difficult to be definitive about the required sample sizes at this stage. However, the following principles will apply,

and we offer past data from similar work as examples to help inform the sample size calculations required here. If new relevant data become available before the experiment is to be carried out, preliminary sample size calculations for the relevant experiments will be revisited to ensure the calculations are based on the most up to date information. Due to the exploratory nature of the work, the experiments will be powered at 80%. In general, the significance level will be 0.05, but where multiple comparisons are required (for example experiments with more than two groups) the family-wise-error-rate will be held at 0.05 by making an adjustment to the significance level appropriate to the number of planned comparisons. All sample sizes will be inflated by a factor of 10% to allow for attrition, for example if animals need to be killed at humane endpoints and therefore cannot complete the study. Where we have used standard deviations from previous studies to inform the calculations, we will increase the standard deviation assumed by 10% to allow for differences in variability across studies.

Protocols 2, 3, 5, 6, 7, & 8 involve parallel group design experiments, with tumour volume post- treatment as the outcome measure. Baseline data for tumour volume will also be measured. Most of these experiments will involve two parallel groups, a control group and an active treatment group.

However, Protocols 2 and 3 govern the testing of heterotopic models screening a wide range of anticancer drug combinations and may include experiments with three or four groups, typically one control group and two or three active treatment groups. All the experiments will be analysed by ANCOVA (analysis of covariance), with treatment group and baseline measurements as covariates and will output estimates of mean tumor volume, adjusted for baseline. Using ANCOVA adjusts for baseline differences between groups and has greater power than a t-test leading to smaller required sample sizes. The sample size calculations for ANCOVA require an assumption of the correlation beween baseline and outcome tumour volume, if this is not available from previous work. Where this is the case, we have assumed a moderate correlation of 0.5. The sample size will then be the number required to detect a difference in tumour size post-treatment between the two groups using ANCOVA adjusted for baseline, with 80% power, alpha of 0.05 and assumed correlation r between baseline and outcome measures, where r is obtained from previous studies or is set at 0.5. For experiments with more than one treatment contrast of interest, the study will be powered to detect the smallest pairwise difference and adjusted for the number of treatment contrasts being studied. It is likely that some treatment contrasts will require large sample sizes; in this case, the study is not powered to detect such a difference, but the results may serve as signals of efficacy and used to help inform future research.

In the following paragraphs, we outline some examples in further detail and the sample sizes can be seen in the Table 1. Protocol 5 will be testing the effect of anti-cancer agents on orthotopic oesophageal tumours. There are few data published in this field about reductions in tumour size using these treatments, so we will base our calculations on published data on human breast cancer organoids in nude mice from Sach et al 2018. In this study, the control group had an average tumour size of 235 mm3, with standard

deviation (SD) of 135 mm3). Average tumour size in the treated group (Afatinib) was 75 mm3 (SD 20 mm3) (appendix 5a). This study will require 9 animals per group, after allowing for 10% attrition. The experiments for Protocols 6, 7 and 8 are similar, and calculations will be conducted as described above, using the most up to date relevant data available to inform the calculations. All assumptions made for such calculations will be captured and available for scrutiny.

Protocols 2 and 3 cover heterotropic models that may require more than two parallel groups. In this case, we will power the study for several independent t-tests and will allow for multiple comparisons by reducing the signficance level for each test to control the total Type 1 error rate.

We will use Protocol 2 as an example of how this will work.

We will use data from a study on the trampC2 prostate model in which the average tumour size in the control group is 208 mm3 (SD 189 mm3). There are three possible treatments of interest; reovirus, PD- 1, and reovirus / PD-1 combined. The mean tumour size and SD for each treatment group are 120 mm3 (SD 111 mm3), 143 mm3 (SD 89 mm3) and 21 mm3 (SD 23 mm3) respectively (appendix 5a). There are six potential treatment contrasts here if every pairwise comparison in included, but the potential effect sizes may be so small in some cases that a) there is insufficient signal of efficacy to warrant further research into that treatment comparison and b) the sample size required to detect such a small difference would be very large (see Table 1). The study will therefore be powered to detect the three potential differences that are of greatest interest. In this study, 18 animals per group would have 80% power to detect the difference between reovirus and the combined treatment, if such a difference exists, and if the standard deviations in the two groups are similar to those seen in Annel. It would also be powered to detect differences between PD-1 and combined treatment, and between the control.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to limit the number of mice used in our experiments we will firstly test our novel anticancer agents both in in vitro cell culture models and using an ex vivo tumour slice model. The in vitro models will allow us to establish the half maximal inhibitory concentration (IC50) for the cell line that we are using and establish the potency and mechanism of action of the anticancer agents that we are studying.

As described above our group has developed a tissue slice culture model system. This system has allowed our group to refine and reduce the number of animals needed for our cancer therapeutic studies. We can obtain tumour tissue from protocol 1 (Standardisation of tumour growth after injection of live tumour cells). After the tumour growth model has been studied and the animal is culled the tumour can be divided into up to 30 slices which allow multiple drugs and viral doses to be tested at any one time. These may give us an indication of possible drug and viral doses for use in protocols 2-3 therefore refining and

reducing the number of animals needed for a project. A further source of in vivo tissue material could be obtained from the PBS control tumours from protocols 2-3. This will therefore maximise the amount of scientific information obtained from each animal used in a procedure. A wide spectrum of treatment types and routes described are used to screen a wide range ofnovel anticancer drug combinations and establish efficacy and toxicity in vivo. We have refined routes of treatments such as IV, IP, injections, infusion, gavage etc based on a comprehensive review of the scientific literature and extensive experience acquired in previous license. Data from this protocol (1, 2 3 and 9) can be used to help design drug dosing regimens and minimise/eliminate adverse effects in orthotopic protocols (4, 5, 6, 7 and 8) within this license. Imaging in vivo will allow us to obtain longitudinal data giving us more information and reducing numbers of animals needed for our studies. Throughout the life of the license, we will analyse the data produced and conduct regular reviews of the literature, enabling us to gain more knowledge to help assess appropriate sample sizes.

Advice has been sought and taken from our University statisticians on the design of all experiments in this license, and this will be an ongoing process throughout the life of the licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All animals used in 1, 2, 3, 6, 7, 8 and 9 protocols will be supplied by registered breeder such as Envigo and Charles River. Only the correct number of animals, will be purchased, based on sample sizes which will be set using power calculation’s. Protocol 4 allows us to breed GAA (L2-IL-1β (Tg[ED-L2- IL1RN/IL1B) mice, for the induction and treatment of oesophageal adenocarcinoma tumours in protocol

5. Breeding of these GAA mice is controlled by a breeding plan, devised by NACWO, NVS and members of our lab, to minimise animal wastage appendix 6. Wherever possible tissue, will be shared within research groups within the university and other institutions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The majority of work carried out under previous project licences has be SC tumour flank model such as C57/BLACK mouse /B16.F10 cells a model of melanoma. These

heterotopic models, which are generally viewed as benign, so are seen to address researchers' obligations to minimise suffering. In this new licence we will still use these heterotopic model to obtain scientific data, but also they can be used to help design drug dosing regimens and minimise /eliminate adverse effects in orthotopic protocols within this PPL.

In this new licence a small number of studies will be carried out using IV injection. Other tumour cells that will be used in either SC or IV tumour flank models include K1735, A375 (melanoma), AY-27, EJ, T24, Ku19-19, 5637, RT112, TCCSUP-1, VMCUB, MB-49 (bladder) DU145, LNCAP, Tramp c2. PC-3, (prostate) SKOV-3, PEA1, 2, (Ovarian) MDA-MB-231 ZR-75-1, MCF7, SK-BR-3, (breast), CT-26, CaKi- 2, HT29, SW620, HCT-116 (colon) PL45, PANC-1, PSN 1, AsPC1, HPAF II, BxPC3 (Pancreatic) A549,

H520 (Lung) LN-18 , U87 (Brain) and Renca (renal). These will be grown in immuno-competent (C57BL/6, BALB/c and C3H/H) or immuno -deficient mice (Nude, Scid). These are examples of possible models to be used, but other models may be investigated either by a comprehensive review of the scientific literature or pilot tumour growth studies (see protocol 1) as recommend by Workman et al 2010 [37]. Pilot tumour growth studies using small numbers of animals can indicate patterns of local and metastatic tumour growth. Any adverse effects due to tumour progression can assess and the clinical score sheet can be adjusted. These studies can help identify the correct humane endpoints.

Data from these studies can help define group numbers in order for experimental time frames.

Based on the data from heterotopic models, we will be able to carry out orthotopic models in such areas as oesophageal adenocarcinoma (protocol 4-5), intra- bone (metastatic) cancer (protocol 6), prostate cancer (protocol 7) and bladder cancer (protocol 8). These models can be used to obtain more clinically translatable data on novel anti-cancer agents.

Protocols 4 and 5 Oesophageal cancer – of which adenocarcinoma (OAC) is the predominant subtype in the United Kingdom - is a highly aggressive malignancy, ranking sixth among all cancers for mortality, with 5-year survival rates of approximately 15%. The TME is a complex interaction between the tumour, host immune cells, fibroblasts, chemokines and other factors such as hypoxia and acidosis. An orthotopic model is the best way of replicating the TME, as the tumour develops within the native organ architecture of the cancer to ensure close similarity to the human TME. The L2-IL-1β genetically modified orthotopic mouse model allows for the generation of OAC in-situ at the squamo-columnar junction of the mouse, as seen in OAC in humans. This model will give us a TME that we can trial novel immunotherapies to advance the care of OAC beyond what has been possible to date.

Protocols 6 and 7 will allow us to study the effect of anti-cancer treatments on two forms of cancer: primary and metastatic cancer. Protocol 7 describes an orthotopic prostate cancer model involving the surgical injection off prostate tumour cells into the anterior

prostate lobe of mice. Protocol 6 With this orthotopic intra- bone (metastatic) model, we will surgically inject cancer cell lines into the tibia, leading to the development of orthotopic metastatic tumours with a clinically relevant TME.

Protocols 8 To obtain the most appropriate microenvironment for transitional cell carcinoma (bladder) cancer, tumour cells must be allow to attach to the bladder lining and grow into the bladder void. This can only be achieved by damaging the bladder layer using this protocol. Implanting tumours into the tissues of origin within the animal results in development of cancer in an appropriate microenvironment which includes for example stromal cell infiltration, including specific immune cells. A major component of our research is to study the role that our novel anti-cancer agents play in immunogenic cell death and modulating the immune system.

Why can't you use animals that are less sentient?

Reliable models of cancer which have been used in peer reviewed studies previously have been proposed. These have led to successful translation into human trials. The mice and rats proposed are representative of the human diseases: the molecular and immunological properties of the tumour cells are assessable in these models, particularly in response to therapy, and closely mirror the human context. The models are also selected on the basis of our previous experience with them, reliability and reproducibility of results, and that the experiments are completed in a short time to minimise distress to the animals (Workman et al 2010).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

There is a wide spectrum of treatment types and routes described in this licence for screening a wide range of novel anticancer drug combinations and establish efficacy and toxicity in vivo. We will be constantly refining routes of tumour implantation/treatments such as IV, IP, injections, infusion, gavage etc based on a comprehensive review of the scientific literature and extensive experience acquired in previous PPLs. An example of the type of well fare refinement we will try to bring in is discussed in Protocol 8 (Treatment of orthotopic bladder tumours with anticancer agents). A major objective of this protocol (8) is to refine our model by trialling replacements for the acid/alkaline wash such as poly-L-lysine or trypsin treatments, to limit the severity of the model. Imaging in vivo will allow us to obtain longitudinal data giving us more information and reducing numbers of animals needed for our studies. The health checks we propose are based on knowledge acquired in previous PPLs and comprehensive review of the scientific literature, these will be under constant review throughout the life of the PPL.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The Workman et al. (2010) guidelines for the welfare and use of animals in cancer research are an invaluable resource, setting out appropriate humane endpoints and

relevant clinical signs to monitor. We will also use the NC3Rs website which provides a number of resources to help with further refinement to our research. This include advice and guidance on common procedures such as blood sampling, which we have used in this application. This website also have species specific pages dedicated to non-aversive mouse handling and genetically altered mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All the members of the lab, who are carrying out in vivo' studies, will attend the Biomedical Research users group meeting at university of surrey. Also a researcher from the team is a member of AWERB of Surrey University. These animal welfare meetings will allow our lab to have an understanding of current 3R issues within the research community. Throughout the life of the PPL, we will analyse the data produced and conduct regular reviews of the literature, enabling us to gain more knowledge to help refine protocols and procedures in terms of the 3Rs.

75. Understanding how climate change affects mammalian immunity and control of infection

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Infection, Immunity, Heat, Mammals, Climate crisis

Animal types	Life stages
Mice	neonate, embryo, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the effect of short term (heat wave) and long term ambient temperature differences on mammalian immune function and its response to infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Global warming is a reality of major concern. Currently, the global average temperature has increased by 1oC as compared to pre-industrial times, with clear effects on entire ecosystems, flooding and natural disasters. Worryingly, by 2050, global temperatures are predicted to have increased by 2-5oC, exacerbating already visible consequences from global warming. In addition we are seeing an alarming increase in heat waves were

temperatures are between 8 and 11oC higher than average temperatures. Rising temperatures impact many aspects of animal life and health, and have lately been shown to severely weaken an animal's ability to fight off infections. If our body's immune system (our defence mechanism against bacteria, viruses and other bugs) cannot fight infections properly, the body becomes vulnerable, leading to severe illness or even death. Thus, global warming could make humans more vulnerable to dying from infections such as the flu. This is particularly concerning as rising temperatures also affect how bugs behave, allowing them to become more dangerous to the body or to spread into new regions. Understanding how higher temperatures weaken the immune system is crucial to design strategies that will help prevent humans from becoming more vulnerable to infections.

What outputs do you think you will see at the end of this project?

We will understand how a rise in temperature affects the immune system and subsequently what impact this may have on our ability to control infections. This will be in the context of acute 'heat wave' type increases in temperature or prolonged exposure to smaller temperature changes of about 2-3oC, mimicking global warming. Thus, we will gather new information in this project, which will be published for the scientific, medical and political communities and support future research opportunities.

Who or what will benefit from these outputs, and how?

In this study, we aim to elucidate how higher temperature can change the ability to defend the body against infections. Understanding the mechanism behind these changes will give an insight into how we may be able to prevent or treat reduced ability to fight infections due to higher temperatures. From the results of our mouse studies, we can get an insight into how humans may react to similar temperature stress. As well as adding to our general knowledge about the effect of global warming on the human body, our data may enable scientists, doctors and politicians to create strategies that help avoid or treat decreased ability to fight infections due to higher temperatures. This could include strategies to improve the effect of vaccinations or the treatment of an infectious disease during a long hot period, especially in developing countries where people cannot always shield from the heat in climate controlled buildings. Finally, demonstrating that global warming will make our immune systems weaker can be used as yet another argument to advocate for policies to combat climate change.

How will you look to maximise the outputs of this work?

This work will be part of a larger collaborative program including programs working on cells, fish infection models and human trials. The results will be shared at the earliest opportunity through publication and at conferences. Large data sets will be deposited on databases for external access. We will disseminate all findings of our studies, including unsuccessful approaches or non-significant data, through publication in peer-reviewed journals, presentation at scientific conferences, and through meetings with other researchers.

Species and numbers of animals expected to be used

- Mice: 9700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To better understand how global warming affects our body's ability to fight infection, we cannot simply start experimenting on humans, but need to use a model system. The more similar the model is to humans, the more reliably we can make conclusions about humans based on the findings of our study. We have selected the adult mouse as a model animal for our work, as it has a fully developed immune (bug defence) system similar to that of humans. In fact, the mouse immune system is made up of the same parts as the human one and uses the same strategies to get rid of bugs, bacteria and viruses in our body. In fact the mouse is also widely used and very well characterised, which means many different scientific tests have been developed for it which we could make use of to investigate in our study. For example, the well characterised genetic code of the mouse, would allow us to later look into the role certain genes and inheritance plays when it comes to vulnerability after being exposed to high temperatures.

Typically, what will be done to an animal used in your project?

Animals in this project will typically be subjected to increased ambient (meaning 'outside') temperatures for a period of time and then infected with a bug (virus / bacterium) or vaccinated. This will involve either an injection, such as intravenously (i.e into a vein) or intranasal (i.e into the nose) and oral gavage (a tube inserted via the mouth into the stomach). Samples of blood will be taken from a tail vein (blood vessel) at predetermined time points. Animals will be monitored during the infection period by weight and in some infections by sampling of faeces. At the end of the experiment all mice will either be humanely killed, or blood collected under deep, terminal anaesthetic where they will be asleep/unconsciousness throughout. The experiment will be typically 1 month long. Some long-term breeding under increased temperature will occur over multiple generations. These animals will then be treated as above.

What are the expected impacts and/or adverse effects for the animals during your project?

As the mice will be infected with bugs they are likely to suffer some discomfort. In wild type animals at standard room temperature all the infections we use cause disease, but do not kill the mice. Mice infected with bacteria will have moderate weight loss of 5-15% over 7-14 days and will have mild clinical signs of infection, slight piloerection (hair standing on end as a sign of pain or discomfort in mice), and hunched walking. With flu infections the

weight loss will be 15-25% less than the starting weight over 10 days and mice may develop increased breathing rates.

With increased ambient temperature exposure, we may expect to see increased weight loss and more pronounced clinical signs. We will have pre-defined end points for our infections to capture animals and minimise any suffering.

Mice that are immunised can show mild signs of discomfort that are fleeting and should last no more than 24 hours. These mice are not expected to show any long term adverse effects.

Keeping animals at temperatures over 30 degrees could increase incidences of poor lactation in breeding females and heighten aggression in males.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice Mild 40%
- Moderate 60%
- Severe 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We only use live mice when we cannot use alternative approaches, such as working with human or animal cells in a dish (these could be blood cells or cancer cells for example that have been taken from patients). It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of the whole animal. Additionally, it is not possible to study the complex interaction between bugs and the host that they infect (e.g. humans) outside of a whole and living animal. We recognise that our mouse model system has limitations and cannot reproduce all the conditions associated with parallel human infections or vaccinations. However, mice have similar immune systems to humans, making observations in mice comparable to those in humans. As mice don’t always react the same way to bugs that attack humans, we use mouse-specific pathogens to evoke a more meaningful response that can be paralleled in

humans. These mouse-specific pathogens include an adapted version of the flu virus that causes similar symptoms in mice to the ones humans experience from the flu, or an adapted bacterium that mimics stomach infections causing diarrhoea etc in humans.

Which non-animal alternatives did you consider for use in this project?

Cells and organoid systems (recreating organs or parts of them from cells in the laboratory, for example part of a gut) have been considered.

Why were they not suitable?

It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of the whole animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

By keeping our experimental conditions well controlled we are able to perform highly reproducible and statistically meaningful experiments using the minimal number of animals. The experimental approaches described herein have been vigorously evaluated over the past two decades. We have access to and have used an experienced statistician to help guide experimental design. Post-doctoral scientists in our group have been on experimental design courses to help understand sources of bias and variation and how best to reduce them. Where possible in our design we blind people to genotype/treatment groups with different people doing infections or analysis. Based on our experience with pathogen infected mice, we use 5-6 mice per group as this is a sufficient sample size given the differences between means and within-group variances we typically observe. We will share any new mouse models we generate with other researchers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have been running these infection models for the last 20 years and during that time we have generated thousands of data points in wild type mice in normal conditions. From this we know what the sources of variations are and have therefore been able to control for them. We have worked closely with Biostatisticians when setting up high throughput screens using these models to reduce the numbers of mice we need to use to get meaningful results. We have used the NC3R's experimental design assistant for work we have done on previous studies and will continue to use it in future studies. The PREPARE

guidelines have also been consulted for formulation of this project, and these will be followed to ensure continued communication between the animal facility and our team.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As part of the work in this program we will use pilot studies to inform us on the control experimental conditions we need to use. These will indicate if we need to alter food intake in control animals, as mice at higher temperature don't eat as much food and if mice at thermoneutrality (29oC) have the same base line outputs as mice at room temperature (21oC) and therefore which mice should be used as controls. We have used the infections in this licence as part of a large screening programme over the last 15 years. The models are set up in a way that allows us to identify differences in test conditions (be that individual gene deleted mice or in this case increased temperature).

The work is also a part of a larger program of work within the department where data will be generated for other experimental models including work in fish and humans. The data generated in these programs will also inform the work herein. Samples collected from any mice as part of experiments planned will be stored long term at -70oC. This will make the samples available for future analysis.

There is potential that other groups may want to use post-mortem tissues that we do not take from mice that have been exposed to increase temperatures.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have established that the mouse can be used to identify bug (also referred to as 'pathogen') and mammalian genes that influence infection and immunity. The wild type parental bugs that we use are almost exclusively disease-causing for the mouse and thus likely to yield important and relevant phenotypes and associated data. Although the pathogens we use are mainly mouse pathogens they are good correlates to human specific diseases. We believe that the similarities in the mouse and human genomes are such that we can infer between the two and we have closer links than ever before with patients in the clinic. Over the years we have gained tremendous experience with our infection models and, through careful observation, we are able to minimise the potential suffering of the animals.

We have been able to identify key clinical signs that indicate illness in infected animals and consequently such animals can be quickly and humanely killed.

Why can’t you use animals that are less sentient?

It is not possible to mimic all aspects of the interactions between the different stages of a real infection process meaningfully outside of the whole animal. It is not possible to study the complex interactions between the host (e.g. humans or mice) and infectious agents / bugs (e.g. the flu virus) outside of whole living animals or in animals that don’t have a mammalian immune system. We recognise that our mouse model systems have limitations and cannot reproduce all the conditions associated with parallel human infections or vaccinations. However, mice have many similarities to humans, including in terms of their immune system. We also use mouse-specific bugs to evoke a more meaningful response that can be paralleled in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are monitored throughout all experiments and we collect daily scores composed of a set of physical signs of illness such as piloerection (raised hair as a sign of pain or discomfort), hunched walk and mobility along with weight loss. The cut-off for these physical signs lies within the guidelines for moderate severity, i.e. loss of pre-set percentage body weight being our main indicator, along with mobility (ability to feed and water). The scoring for piloerection etc. are also used as secondary indicators. My team are experienced in animal infection models and are trained to the high standards that I expect. The technicians that work in our holding facility and do the majority of the animal husbandry will also be trained by my team and will communicate abnormal behaviours in the mice early. At our establishment we have dedicated Named Animal Care and Welfare Officer who are impartial and can give advice/ make decisions on animals that lie outside of the normal adverse effects expected for the infections outlined within this project.

Potential refinements include increased monitoring if test animals show earlier clinical signs or weight loss. We will give wet mash food to animals that lose more weight quickly. Floor food will be given to animals that are to be infected to limit weight loss from the start of the infection.

Litters from breeders at higher temperature will be monitored closely for development as temperature may affect lactation in mothers. Any evidence in pups not developing or litter losses will be discussed with NVS and appropriate actions taken.

All animals will be given environmental enrichment and be socially housed to encourage natural behaviours and reduce stress. Males will be monitored for increased aggression and additional enrichment added to combat or appropriate splitting of fighting animals if needed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use guidance from the NC3Rs website and the Laboratory Animal Science Association (LASA) to ensure experiments are conducted appropriately. In particular we will follow the 'Guiding principles on good practice for Animal Welfare and Ethical Review Bodies'.

We will follow the PREPARE guidelines for planning experiments and will follow the ARRIVE guidelines reporting of results.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep informed on advances through the NC3R's website, Norecopa website, our establishment website and newsletter. We will discuss any advances with the relevant people at our establishment and implement them accordingly.

76. Early safety assessment, investigatory and efficacy studies

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

safety, pharmacology, drug-development, efficacy, physiology

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to provide a service to pharmaceutical companies who required an assessment of the safety of their potential new medicines on vital body organ function (heart, lungs, central nervous system (CNS)). The project will also allow the efficacy of some potential new medicines to be assessing on behalf of Sponsor companies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Before regulatory authorities will grant permission for testing potential new medicines to humans, they require the safety of the compound to be assessed in animals. Standard toxicology and safety pharmacology studies are performed to fulfil regulatory requirements prior to first-time-in-man clinical trials. If safety liabilities are identified in these regulatory studies, options to change the chemical scaffold of the molecule at this late stage are limited. This can result in wasted money, resources and animals on compounds which are not ultimately suitable for development as potential medicines.

Consequently, there has been a drive to incorporate safety assessment earlier in the drug discovery/development process when studies can be performed to identify potentially undesirable pharmacodynamic effects or overt toxicities and thus identify unsuitable targets and/or eliminate unsuitable drug candidates. At this earlier stage, compound series are still being identified and optimised and modifications may be made more easily to improve safety. Whilst early in vitro safety screens have become more widely used, early in vivo screens in rodents are also often employed within the discovery phase to investigate potential undesirable effects on physiological function. Effects on vital organ systems (cardiovascular, respiratory, CNS) can be made using specialist equipment.

Use of rodents is beneficial since compound requirements are low and synthesis is expensive at this early stage.

In addition to their use in early screens, rodent models are also used as investigational follow-up studies to evaluate pharmacodynamic or pathophysiological effects of test substances observed in previous pre-clinical or clinical studies. Studies can be performed to investigate the mechanism of an observed toxicity.

Anaesthetised rodent models also lend themselves to early efficacy assessments of candidate compounds. Efficacy is generally assessed by administration of a stimulatory substance in addition to the candidate drug. Body fluids, tissues or organs can then be collected for measurement of relevant biomarkers.

Under this licence new medicines (e.g. cancer therapies, medicines for gastrointestinal disorders or CNS diseases) will be tested in small animal protocols with the aim of identifying undesirable effects on the cardiovascular, respiratory or central nervous system. The medicines will usually be small molecules but, occasionally, peptides/proteins, antibodies and antibody drug-conjugates may be tested. Both early screening studies assessing effects of test compounds on vital organ systems and investigational studies to determine mechanisms of observed effects will be performed. Studies to assess the potential efficacy of test compounds will also be conducted. This information helps Sponsors prioritise the most promising candidates for further development, eliminate unsafe medicines and make necessary changes to candidate

molecules to make them safer for animals in future animal testing and for humans in clinical trials.

The following references support the need for the work related to this project:

Anon. (2001). ICH S7A: Safety Pharmacology Studies for Human Pharmaceuticals.

Available at: https://www.ich.org/page/safety-guidelines

Anon. (2005a). ICH S7B: The Non-clinical Evaluation of the Potential for Delayed

Ventricular Repolarization (QT Interval Prolongation) By Human Pharmaceuticals.

Available at: https://www.ich.org/page/safety-guidelines

What outputs do you think you will see at the end of this project?

The project will generate new information on the safety of many potential candidate medicines which will be used by the Sponsors in their drug discovery and development programmes. Examples include quantitative profiles of the effect of potential medicines on:

- arterial blood pressure, heart rate, ECG (electrocardiogram) parameters, contractility of the heart,
- brain activity via EEG (electroencephalogram) recording
- breathing rate, breathing volume (tidal volume)
- body temperature and activity

By recording additional physiological parameters, using surgical interventions or using blocking drugs, the project will also generate data outputs that may help Sponsors determine:

- the mechanism of any adverse effect (e.g. is the decrease in blood pressure due to dilation of blood vessels or effects on the heart)
- the site of action of an adverse effect (e.g. via action on the brain or via an action in the periphery)

The project may also generate new information on the efficacy of potential new medicines by measuring relevant biochemical markers in certain body fluids (e.g. urine, blood, plasma).

Other outputs include:

- information on the tolerability of the test compounds
- information on the pharmacokinetics of the test compounds (e.g. maximum concentration [Cmax] , time of maximum concentration [Tmax], half life, area under the concentration vs time curve [AUC]).

Who or what will benefit from these outputs, and how?

Outputs from early rodent safety or efficacy screens will help Sponsors improve their compound selection process during the drug discovery phase. Studies performed on candidate, or competitor compounds (compounds already on the market or in development by other companies) will help to assess the safety risks that may be associated with the selected drug target and also determine whether the pharmacological class or chemical series has inherent safety liabilities associated with it. This should promote the development of compounds with a more acceptable pharmacological and toxicity profile.

By testing the effects of test compounds in the therapeutic range and above (supra-therapeutic), the data outputs will help Sponsors assess the onset, dose-response and reversibility of any adverse effect of their compounds. This will help with stop/go development decisions and help select the most appropriate candidate for further development. Progression of safer drugs will reduce the severity and/or incidence of adverse effect in regulatory preclinical animals studies and will contribute to the safety of clinical trials, reducing the chances of side effects in healthy volunteers and patients. It is expected that these benefits would be realised within the lifetime of this licence since most compounds tested would be preclinical candidates which, if successful, could be expected to progress into clinical trials within 2-4 years.

Identification of safety issues early in the discovery phase will provide the opportunity to investigate the mechanism of the toxicity and its relevance to man before clinical trials are considered. A mechanistic understanding of any adverse effects may help Sponsors assess the risk/benefit of further development. Identification of mechanisms of toxicity that may not be relevant to humans may allow development of medicines that would otherwise not have been available. Indeed, such mechanistic investigations are often requested by regulatory agencies. Where a compound with a known safety liability progresses to clinical trials, a mechanistic understanding of the liability, gleaned from prior preclinical studies, can help improve the clinical risk management plan. It is expected that there will be reduced attrition of potential medicines in development, as a result of better early selection, and faster delivery of new medicines to patients.

How will you look to maximise the outputs of this work?

Where possible, we will publish data from the studies performed, in collaboration with Sponsors, in high quality journals. There are often restrictions surrounding publications of live project compounds but opportunities for publication often exist once approval is granted or on data for compounds that do not proceed to clinical trials.

Any refinements that we make to our protocols and animal models will be presented at a relevant scientific meeting (e.g. Safety Pharmacology Society) and also disseminated by

publication in a relevant journal (e.g. Journal of Pharmacological and Toxicological Methods; IAT Journal) so that other may learn from our experiences.

Species and numbers of animals expected to be used

- Mice: 375
- Rats: 1025
- Guinea pigs: 245

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The rat is generally the rodent species of choice in drug safety testing and some efficacy testing. The physiology of the cardiovascular, respiratory and central nervous systems has been well characterised in this species. There is a wide knowledge of the response of rats to various chemical entities and many drugs show similar effects in rats and humans. Rats are therefore considered an appropriate species to use for evaluating the potential adverse effects to humans of new drugs on vital organ system function.

Mice may be used if for some reason they are a more appropriate species than the rat e.g. for drug kinetic reasons or if their expression of a drug target is more similar to humans than rats. Mice are often used in efficacy or toxicology studies for reasons such as this and adverse effects previously observed in these studies may need to be investigated in the same species.

Guinea pigs are used preferentially for studies on cardiac electrophysiology as the expression of ion channels in the heart is more representative of the human situation compared to rats and mice and it has been shown to be a predictive species for drug effects in humans. Guinea pigs are also a more appropriate species to use in respiratory studies if the compound is suspected to cause airway hypersensitivity responses, since the receptor pharmacology more closely resembles that of the human airway. Use of a small animal model results in a low compound requirement which is of benefit in early drug development.

Adult animals will generally be used since most drug tested will be for indications in adult humans.

Typically, what will be done to an animal used in your project?

Animals used in this project may be used in terminal anaesthesia protocols, recovery anaesthesia protocols or in conscious animal studies.

Terminal anaesthesia: In terminally anaesthetised studies an animal will be anaesthetised and surgically prepared to allow recording of various physiological parameters. This includes:

- placement of cannulae in blood vessels to measure blood pressures and for intravenous (i.v.) dosing of the test compound
- placement of ECG leads under the skin to measure the electrical activity of the heart
- measurement of respiratory parameters using a flow transducer, or accelerometer placed on the skin. The breathing of some animals may be controlled by artificial ventilation
- placement of biopotential leads on the cranium to record an EEG

Following surgery, the animals will be allowed to stabilise and then one or more doses of the test drug will be administered to assess its effects over time on the physiological parameters. The animals remain anaesthetised for the whole experiment and recordings may be made for up to 5h after which time the animal is humanely killed without regaining consciousness.

Recovery anaesthesia: Recovery anaesthesia is required in some studies to surgically implant telemetry devices. These devices allow the following physiological signals to be measured:

- arterial blood pressure via a catheter implanted in the aorta, femoral or carotid artery
- ECG via biopotential leads positioned on the xiphoid process and subcutaneously or intramuscularly
- EEG via biopotential leads positioned and secured on the cranium

The body of the implant device is usually secured in the peritoneum or under the skin. Animals are monitored closely throughout and after the surgery and are given medication to minimise the pain that may be experienced afterwards. Once they have recovered from surgery (after at least 2 weeks) they may be dosed (usually orally) with test drugs and the effects of the compound on physiological parameters over time is recorded remotely without having to disturb the animals. When a test compound needs to be infused intravenously recovery surgery may also be required to implant a cannula in a vein. This is better option than using a temporary intravenous catheter which would require long-term restraint and stress. Animals implanted with telemetry devices are usually used and then re-used for other drug assessments following a washout period.

Conscious animal studies: To assess the general effects of test compounds on the central nervous system, normal (non-implanted) animals are dosed with a test drug and the effects on the animals' behaviour are observed in their home cage, in a separate observation cage or whilst being handled. Conscious animals can also be placed

temporarily into whole body plethysmography chambers (for up to 8h) in which they can move freely and which allow their breathing to monitored non-invasively. This detects effect of the test drug on the lungs and the respiratory system.

In most studies, blood samples are taken from the animals to measure how much of the test drug is in the body. In terminally anaesthetised animals this is usually taken from a cannula in a blood vessel. In conscious animals, the blood is taken via a tail prick and the number of samples and volume of blood taken are minimised.

What are the expected impacts and/or adverse effects for the animals during your project?

We do not expect to see many adverse effects due to the dosing of the test compounds since Sponsors will usually have prior in vivo data on the test compounds such as results from efficacy studies, pharmacokinetic studies or preliminary toxicology studies, including maximum tolerated dose (MTD) studies. When there is no prior tolerability information, a pilot study will be performed in a small number of animals to assess the tolerability of the test compound and possible adverse effects. This will ensure safe and relevant doses are administered to the animals in successive studies. To ensure a thorough assessment of safety at therapeutic and supra-therapeutic doses it may be necessary to dose up to or near the MTD (the MTD being a dose that may cause target organ toxicity but is anticipated to be tolerated and will not jeopardise the study by causing morbidity or mortality).

Some animal may therefore experience adverse effects as a result of the dosing of test drugs such as weight loss, piloerection (raised fur), hunched posture and subdued behaviour. When they occur, the adverse effects of the test drugs will be expected to occur around the time of the maximum drug concentration in the blood and will be of short duration dissipating as the drug is cleared from the body. Humane end-points are applied as necessary to minimise the suffering of the animals.

Adverse effects may also occur as a result of the dosing procedure. Animals will be dosed by routes similar to those used in humans, e.g. by mouth (orally) or by injection (e.g. intravenous, subcutaneous) and these require short-term restraint or confinement. When dosing, animals will be restrained and the drug administered directly into the stomach via a syringe and gavage tube or into a vein. This may cause a period of discomfort whilst the procedure is being performed. Transient discomfort may occur during the sampling of blood, usually via a tail prick, which is required in most studies to determine the amount of test drug in the body.

Some animals may experience short-term discomfort following surgery to implant telemetry devices or to implant intravenous catheters for longer duration injections. Subdued behaviour and some signs of discomfort may be seen in the days after surgery and weight loss is often observed during the week post-surgery. Analgesics and anti-inflammatory drugs are given to relieve post-surgical pain.

Some animals may experience adverse effects due to single housing and lack of social contact. Single housing is usually required after recovery surgery for 1-2 days, during some telemetry studies when intravenous dosing is required and also during conscious assessment of lung function (for up to 8h).

Singly housed animals will be housed adjacent to each other animals so they have visual contact at all times.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?
Rats: Non-recovery: 85% Moderate and/or Mild adverse effects 15%

Guinea Pigs: Non-recovery: 85% Moderate adverse effects 15%

Mice: Non-recovery: 70% Moderate adverse effects 30%

The majority of animals used in this project will be used in non-recovery procedures only.

Approximately 15% of rats and 30% of mice and 15% guinea pigs may experience moderate adverse effects due to surgical implantation of telemetry devices or vascular catheters. Mild adverse effects are likely to be experienced during the subsequent dosing of test compounds and blood sampling although occasionally these could be moderate.

Some rats, not implanted and used for conscious respiratory or CNS assessments, may experience mild effects or moderate adverse effects due to the dosing of test drugs and blood sampling.

Examples of mild adverse effects include slight weight loss, partial piloerection (slight raised fur), transient hunched posture and subdued behaviour or transient discomfort due to dosing restraint or blood sampling. Examples of moderate adverse effects include more marked weight loss, marked piloerection (marked raised fur), intermittent hunched posture, subdued behaviour even when provoked, intermittent tremors (marked but not continuous).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Sponsors will assess efficacy in available in vitro and ex vivo tissue systems and will assess safety using non-animal methods where possible. However, to understand if a potential new medicine has the desired effect in vivo or to investigate how it affects the function of vital organs, either directly or indirectly, it is still necessary to use a whole animal in which all organ systems are present and their physiology is intact. This is in accordance with international regulatory guidelines that specify the generation of safety data in two species, one of which must be a rodent.

Which non-animal alternatives did you consider for use in this project?

It is likely that in vitro/ex vivo assays will have been used to select the compounds that will be tested in animals in this licence as part of the usual screening cascade that occurs in the discovery and optimisation stages of drug development. Non-animal alternatives such as these have already reduced the demand for some of the projects conducted under this licence. For example, use of stem cell-derived cardiac myocytes enables Sponsors to perform some early cardiovascular screening (assessing QT liability) without the use of animals. Selection between candidates will now usually be made based on these in vitro results, rather based on in vivo data from multiple studies, and often only the final selected compound is tested in animals. These in vitro data will be used alongside any in vivo data generated under this licence to form an integrated risk assessment used ultimately to minimise the risk to volunteers and patients in clinical trials.

Why were they not suitable?

Whilst in vitro/ex vivo assays are used by Sponsors in the screening and selection process, to understand how a test compound may affect the function of vital organs, either directly or indirectly, it is necessary to use a whole animal in which all interrelated organ systems are present and their physiology is intact. This is in accordance with international regulatory guidelines that specify the generation of safety data in two species, one of which must be a rodent.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

As a Contract Research Organisation, the number of animals used will primarily be based on the number of studies performed for Sponsors. The estimates are based on actual numbers used in previous years on a similar project licence (30/3403).

2 non-recovery mice studies per year are likely with a group size of n=4-6 and 2-3 groups per study (approximately 125 over 5 years).

30 mice are likely to be used in conscious telemetry, respiratory or CNS studies per year (usually n=4-6 per study) (approximately 150 over 5 years).

20 mice are likely to be used in tolerability/pharmacokinetic studies per year (usually n=2-6 per study) (approximately 100 over 5 years).

10 non-recovery rat studies per year are likely with a group size of n=4-6 and 2-3 groups per study (approximately 625 over 5 years).

50 rats are likely to be used in conscious telemetry, respiratory or CNS studies per year (usually n=4-6 per study)(approximately 250 over 5 years).

30 rats are likely to be used in tolerability/pharmacokinetic studies per year (usually n=2-6 per study) (approximately 150 over 5 years).

3 non-recovery guinea pig studies per year are likely with a group size of n=4-6 and 2-3 groups per study (approximately 190 over 5years).

8 guinea pigs are likely to be used in conscious telemetry studies per year (usually n=4-6 per study) (approximately 40 over 5 years).

5 guinea pigs are likely to be used in tolerability/pharmacokinetic studies per year (usually n=2-6 per study) (approximately 25 over 5 years).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Study directors working under this licence have extensive experience of designing these study types and aim to use the minimum number of animals that allows the study to reach its objective. We also have an on-going collaboration with statisticians in a partner company who perform statistical health checks and advice on the design of the study, the number of animals per group, randomization and the method of statistical analysis.

Prior data analysed by our partner, and in preparation for publication, has shown that test drug effects on physiological parameters can be detected with acceptable sensitivity in small groups of animals (n=4-6) in non-recovery studies. Increasing the group size to n=8 improves the sensitivity of detection only marginally. The good sensitivity with small group sizes is likely to be due to the fact that the measurement of safety endpoints and efficacy biomarkers is conducted under terminal anaesthesia which may reduce the variability. We will continue to utilise this technique so that animal numbers are reduced.

Our previously published data has also shown that small drug-induced effects on physiological parameters can be detected with acceptable sensitivity in conscious animals using telemetry with a group size of n=6. Again, increasing the group size to n=8 has only

marginal benefits in terms of the power of detection. We routinely use optimal data summarising techniques (e.g. superintervals) in conscious animal studies which contributes to the good sensitivity.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All studies will be designed to maximise the amount of useful data obtained using from each animal. Pilot studies will also be used where necessary to ensure that safe and optimal doses are selected for the main studies. This will prevent studies having to be repeated, and animals used unnecessarily, due to poor dose selection.

Assessment of the safety of a test drug usually requires a dose-response to be investigated (at therapeutic levels and above). In non-recovery studies and conscious animal studies multiple doses of test drug will be tested in the same animal where possible rather than using separate animals for each dose. In conscious studies this will be achieved by using cross-over designs and appropriate washout periods between doses.

It may be possible in some studies to incorporate physiological endpoints from multiple organ systems into the same study, potentially negating the need for separate studies. E.g, cardiovascular and respiratory endpoints in a single terminally anaesthetised study; cardiovascular and EEG recordings in a single conscious telemetry study.

Blood sampling may also be included alongside the safety assessment, especially in non-recovery studies, to allow the effects of test drugs to be correlated to the concentration of drug in the blood/plasma. This may obviate separate satellite pharmacokinetic animal studies. Samples of tissues or body fluids taken at the end of the study may be frozen and stored for further analysis.

In some non-recovery studies, it may be possible to obtain efficacy data alongside safety data in a single study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Protocol 1: Safety and efficacy assessments in terminally anaesthetised rodents

The majority of animals used in this project will be used in the non-recovery terminal anaesthesia protocol. Use of terminal anaesthesia minimises pain, suffering and distress to the animals. Surgical preparation will be done aseptically. Whilst the anaesthesia itself may blunt certain physiological reflexes (although this can be mitigated by appropriate choice of anaesthetic), this can make the model more sensitive in detecting drug-induced effects which is often advantageous if used during the early discovery drug development phase. Pre-dosing of conscious animals prior to the terminal phase may be required in some studies (approx. 10% of terminal studies). If any pre-dosing is required, the doses used will usually have no adverse effects having been tested previously either by the Sponsor or in a pilot study (this licence). Occasionally doses administered may cause mild or moderate adverse effects. This is more likely to occur when assessing doses in excess of the predicted therapeutic range which is a requirement for safety testing.

- Protocol 2 Tolerability/Pharmacokinetic studies

In most of the main studies conducted under the other protocols, information about the tolerability of the test compound will already exist and doses will be selected accordingly. Where prior tolerability data is absent, a pilot study will be performed ahead of the main study in separate groups of animals using an ascending dose design. Suffering of the animals will be minimised by using a small group size for assessing each dose (usually n=2) and using small dose escalations, escalating only if the previous dose was tolerated. The results of this study will enable confident setting of tolerated doses for the main study which will minimise suffering of the larger group of main study animals. Animals may also be dosed to assess the concentration of test drug in the blood/plasma (pharmacokinetic study). This will usually be performed to support a separate safety study conducted under this licence. Assessing the pharmacokinetics in a separate group of animals is often preferable if the amount of blood sampling required would compromise the safety data. The number of blood samples and the volume of blood taken will be minimised by using a micro sampling technique, where possible, which also minimises the distress and restraint required.

- Protocol 3 Monitoring of physiological parameters in conscious rodents

The gold-standard method of assessment of drug-induced changes in cardiovascular parameters and the electroencephalogram (EEG) is with the use of conscious freely-moving animals using implantable telemetry technology. These techniques, which will be employed under this licence, allow the assessment of drug effects in an unperturbed physiological system with all reflex mechanisms and regulatory systems intact. For studies using telemetry, animal suffering related to the implantation of the telemetry device will be minimised by the use of aseptic surgical techniques with appropriate recovery anaesthesia and analgesia and post-surgical monitoring.

The amount of animal restraint required will be minimal on oral dosing telemetry studies (estimated to account for 70% of telemetry studies) and any blood sampling will be limited (due to its adverse effects on the recorded parameters). When test compounds need to be

administered by intravenous infusion, it will usually be more appropriate to surgically implant a venous cannula for dosing (approx. 20% of telemetry studies). This allows the animal to move freely whilst being dosing in its recording cage and minimises the suffering that would otherwise have been caused by prolonged restraint. The implanted catheter is usually connected to a skin button and swivel and tether system which necessitates single housing for the duration of the dosing and recording. Single housing is necessary to prevent possible damage to the equipment and cannula by cage mates which could result in a failed experiment and euthanasia of the animals. Environmental enrichment such as bedding and chew-sticks may still be provided to alleviate stress induced by single housing and the use of protective caps on the skin buttons enables group housing during non-dosing days. Alternatively, for short intravenous administrations, it may be preferable to physically restrain the animals in a restraining tube and insert a temporary catheter into a lateral tail vein (approx. 10% of telemetry studies).

- Protocol 4 Behavioural testing in conscious rodents

The Irwin test is a standard method for assessing the effects of a test compound on the central nervous system (CNS). It is the most suitable procedure since it permits evaluation of behavioural, neurological, autonomic and peripheral nervous effects with non-invasive monitoring and minor animal handling. No surgical procedures are necessary and animals can be group-housed for the majority of the study.

Animal suffering should be restricted to dosing, limited blood sampling and potential adverse effect of the test compound.

- Protocol 5 Respiratory assessment in conscious rodents

A standard method of assessment of respiratory parameters is in conscious freely-moving animals using plethysmography. This technique measures respiratory parameters indirectly but it is advantageous since it is non-invasive and the animals are not required to be restrained during recording. Some suffering may, however, be caused by the stress of single housing required within the plethysmography chambers. Pair-housing is not possible during respiratory recording as the pressure signal within the chamber would be affected by the respiratory pattern of the companion. Suffering will be minimised by group-housing when not having measurements taken and limiting the time spent in plethysmography chambers to a maximum of 2h pre-dose and 6h post-dose which should allow sufficient time for the onset and offset of potential drug effects to be recorded. The size of the chambers allows for freedom of movement of the animal and wet food is placed into the chamber for recording periods >4h post-dose as it has been shown to reduce body weight loss. Chambers are made from transparent perspex and multiple chambers will be arranged so that animals are within sight of other animals under test. Animal suffering may also be caused by dosing, blood sampling and potential adverse effect of the test compound.

Why can't you use animals that are less sentient?

Adult rodents are used standardly for the assessment of safety or efficacy of new medicines according to international regulatory guideline since the physiology of their cardiovascular, respiratory and central nervous systems is well characterised and is similar to humans. There is a wide knowledge of the response of rodents to various chemical entities and many studies have shown concordance of drug effects on physiological function between rodents and humans. Rodents are therefore considered an appropriate species to use for evaluating the potential adverse effects of new drugs on vital organ system function. Less sentient invertebrates exhibit more differences in physiology compared to humans and are less likely to predict the potential effects of a test drug in humans. The majority of studies performed under this licence will however be conducted under terminal anaesthesia reducing animal sentience.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Most procedures will be conducted under non-recovery anaesthesia, minimising harms to the animals.

Animals will be group housed as standard and provided with specific materials to provide enrichment. Rats are housed in large double-decker IVC cages, guinea pigs in floor pens and mice in standard IVC cages. We also have a socialisation programme in place where rats are housed in larger groups in a multi-storey playpen for periods of time. This will be utilised for animals that are kept for longer periods of time (e.g. re-used telemetry animals). Any periods of restraint or single housing required during a study will be kept to the minimum required to achieve the study objectives.

Where prior data does not exist, pilot studies will be used to assess the tolerability of any test drug that is required to be given to conscious animals in a study. Doses that cause some adverse effects may be required to be given to animals so that a full dose response safety profile is explored. In all studies, adverse effects experienced by the animal and the number of animals per group will be kept to the minimum to achieve the objectives. Where animals receive multiple doses of a test medicine, sufficient recovery time will be allowed between successive doses. Dosing will be conducted by trained licensees and flexible oral dosing catheters will be the default catheter that is used for any oral dosing.

Capillary microsampling will be used preferentially in all studies when blood samples are required. This technique reduces animal suffering since the volume of blood collected per sample (usually approx 30uL) is smaller than standard samples and the collection process is more rapid and requires less animal restraint. Sampling can usually be performed by one person with the animal being held in one arm.

Appropriate anaesthetics and analgesics will be used during and after any surgical procedures required. Peri and post-operative monitoring is performed closely using a detailed score sheet immediately after surgery for 3-5h with additional checks as required. Enhanced welfare checks with pain scoring are performed for a week after surgery

followed by standard daily welfare checks. Animals are expected to make a rapid and uneventful recovery from surgical implantation of telemetry devices. Animals will be humanely killed in the event of any unexpected post-surgical complications that cannot be easily remedied using only minor interventions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the advice of Procedures with care website for dosing and blood sampling http://www.procedureswithcare.org.uk/

Dose volumes will be used according to standard published guidelines (Morton et al., 2001; Diehl et al., 2001; LASA Good Practice Guidelines, 1998)

Standard published guidelines will be followed for blood volume removal (Diehl et al., 2001, NC3Rs website) and capillary microsampling will be the default method used.

Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C. A good practice guide to the administration of substances and removal of blood, including routes and volumes. J Appl Toxicol. 2001 Jan-Feb;21(1):15-23.

LASA Good Practice Guidelines Administration of Substances (Rat, Mouse, Guinea Pig, Rabbit). Series 1/Issue 1 – October 1998
http://www.procedureswithcare.org.uk/lasa_administration.pdf

Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, Verschoyle R, Westall L, Wilson AB; Joint Working Group on Refinement. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab Anim. 2001 Jan;35(1):1-41.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive updates from the NC3Rs and read animal science journals (IAT Journal, Laboratory Animals) which are good sources of useful information on animal welfare. M Skinner is a member of the University 3Rs committee. We implement any appropriate changes to methodology that will have positive impact on the animals' welfare.

We also strive to continually improve the welfare of animals by making refinements in our facility and publishing outcomes. Examples under a previous licence include the used of double-decker cages for rodent telemetry and the use of a rodent socialisation cage.

77. Cellular and molecular mechanisms of the mouse embryo development

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

mouse embryo, development, cell identity, stem cells, embryonic stem cells

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project seeks to understand early mammalian development with a special focus on how the different cell types of the embryo are formed and organised in the early embryo.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Approximately 50% of human pregnancies fail at this early stage of development and we know very little due to the inaccessible nature of the human embryo at these time points. Therefore, gaining a greater understanding of how early mammalian development occurs will bring us insight into this significant medical problem. These studies will provide us with insights into how the major cell types of the embryo - the supporting placenta and yolk sac, and the embryo itself – are formed in the first week of development and importantly how

and why mistakes in this process lead to loss of the embryo. We carry out work in the mouse system as the mechanisms and biology are likely to be directly relevant to human development and to understanding how pregnancies fail at these early stages. In addition, stem cells, which can be cultured in a dish, resemble the early cells of the embryo. As such, understanding the mechanisms that drive different cell identities in the embryo is also crucial for expanding our knowledge of how to direct stem cells to develop into the different cell types of the body. This will be critical to develop future approaches for regrowing, repairing, or replacing damaged or diseased cells, organs or tissues.

What outputs do you think you will see at the end of this project?

This project aims to answer outstanding questions in developmental and stem cell biology. The Principal Investigator and her research group are well known for running cutting-edge research, which is consistently published in high-impact journals and presented at conferences.

Expected outputs:

- publications in high-impact scientific journals
- conference presentations and papers
- new information in the field
- new experimental methods and approaches that could be used by other research groups in the future
- fostering of new international collaborations that will allow new approaches to current and future scientific questions
- training of scientists in responsible use of animals in research and mammalian embryology techniques
- data to support future funding applications and potential patent applications

Who or what will benefit from these outputs, and how?

Understanding development and cell identity in the embryo has many downstream benefits. In the short term it will improve knowledge of the mechanisms the early embryo uses to become specialised and grow. This knowledge will help us improve methods for working with, regrowing, repairing, and replacing damaged or diseased cells in the early embryo, which will likely impact in vitro fertilisation techniques. It will also impact on efforts to grow embryo-like structures using stem cells, which have huge potential to contribute to our understanding of development and to reduce the use of animals in research.

The immediate beneficiaries of these outputs will be the scientific community. Publications from this project will be published throughout the period of the project and scientists studying development, stem cell biology, reproduction, regenerative medicine and related fields will be the predominant beneficiaries of these publications.

In the medium to long-term, this project will expand our knowledge of how and why pregnancies fail. This will likely lead to improved methods for embryo culture, fertilisation,

transfer and success in reproductive technologies, including fertilisation in the dish. We also expect that improvement of embryo-like structures development from stem cells will replace the use of animals in many areas, e.g. drugs and other chemical compounds testing or assessment of gene function during the development. Data obtained will also improve knowledge of the stem cell-based therapies.

How will you look to maximise the outputs of this work?

- The Principal Investigator and the research group have several established national and international collaborations and are setting up new collaborations on an ongoing basis to maximise impact and dissemination.
- The outputs of this project will be presented at scientific conferences and published in high impact scientific journals to aid in knowledge dissemination. All publications will be open access.
- Dissemination of any approaches (successful or unsuccessful) and/or data (significant or insignificant) we may encounter in the duration of the project.
- The Principal Investigator will continue to recruit undergraduate and postgraduate students, as well as postdoctoral scientist to work on this project. These lab members will receive extensive training in mammalian embryology and the respectful use of animals in research. This knowledge and experience will be disseminated further as these scientists progress in their career and move between different laboratories and groups both academic and industrial.

Species and numbers of animals expected to be used

- Mice: 43 675

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our group is investigating the mechanisms of mammalian development, from fertilization until the formation of the recognisable body after implantation in the womb. Mouse embryos are crucial in developmental biology research and are an excellent model to study events occurring during mammalian development, including many aspects of human development. Methods for mice breeding and maintenance, mouse embryo production, recovery and culture are well established and optimised, ensuring refinement and reduction in animal use. Our group has extensive experience with mouse embryology at pre- and early post-implantation stages.

Ultimately, we are interested in understanding early human development and disease. However, the use of human embryos is technically and ethically challenging. Therefore, the mouse embryo, which undergoes a similar developmental trajectory and allows for

experimental manipulation is an excellent organism with which to carry out this research to investigate aspects of human development. The mouse represents an excellent balance between similarity to the human, size, experimental ability and cost. Our laboratory has a successful track-record of utilising mouse research to investigate and understand aspects of human embryo development.

It is important to add that, part of our scientific focus is the optimization of methods to create mouse embryo-like structures from mouse stem cells. Our success has led to a partial replacement of mouse embryos. However, these models do not yet fully capture the complexity of the mouse embryo, and therefore, it is, as yet, impossible to replace the use of mice in our current research.

Typically, what will be done to an animal used in your project?

Mice are used to generate embryonic tissue in our project. To obtain embryos, female mice will be administered two hormone injections (around 48 hours apart) to stimulate egg production and release. Hormones will be injected intraperitoneally (i.e. into the body cavity) or subcutaneously (i.e under the skin). This procedure, called superovulation, increases the yield of released oocytes, decreasing the number of mice needed to generate a set number of embryos for research. Subsequently, female mice will be placed with male mice for mating to fertilise the released oocytes. Following successful mating, embryos are obtained after humanely culling the dam and dissecting out the developing embryo.

We also plan to produce novel genetically altered mouse lines (without harmful effects), which help us understand the effect and role of specific genes. This procedure involves surgical or non-surgical transfer of genetically altered embryos into the womb of a female mouse, which in turn develop as normal pregnancies. Non-surgical embryo transfer will be used whenever possible as more refined method. However, in cases where it is not possible (e.g. transfer of embryos at very early stages, repetitive problems with non-surgical transfer efficiency that would not result in positive outcome), surgical transfer will be used. Surgical embryo transfer involves a minor surgical procedure performed under anaesthesia to transfer genetically altered embryos into the womb. During surgical embryo transfer mice are unconscious throughout the procedure and will experience short-lived post-operative pain and discomfort. The procedure will be performed in aseptic conditions to reduce risk of infection. To minimise any pain or distress, a range of available analgesia methods will be used. During non- surgical embryo transfer, embryos will be inserted directly into the uterus with a special device without any surgical intervention. Animals experience mild transient discomfort and no lasting harm. For embryo transfer (non-surgical and surgical), female mice are mated with sterile males to induce a pseudo-pregnant state, which is essential for creating a womb that allows the transferred embryo to develop. It is planned to use naturally sterile males (genetically altered) by default as more refined solution. However, we predict that due to unexpected events (e.g. problems with availability), we may need to use sterile males generated by performing a vasectomy, where the tube carrying sperm is cut. We expect that this would be not more than 10% of

sterile males used. These mice will experience minor post-operative pain and discomfort. This procedure will be also completed under general anaesthesia so that the animal will remain unconscious, and in aseptic conditions, to reduce risk of infection. To minimise any pain or distress a range of available analgesia methods will be used.

Following birth, the mice with the genetic alteration are maintained and bred in order to keep the new genetic alteration and establish a mouse colony. Subsequently, the new mouse line would be used to obtain embryos for the experiments as described above.

What are the expected impacts and/or adverse effects for the animals during your project?

For the hormone injections, the doses and routes used are well validated and are not anticipated to produce any adverse effects, beyond transient discomfort due to the injections, which will be intraperitoneal (i.e. into the body cavity) or subcutaneous (i.e under the skin).

Breeding and maintenance of genetically altered animals will have only minor or no effects that are more than minor or transient - animals are not expected to experience harmful phenotypes due to the genetic alteration, i.e. effects that would result in pain, distress, suffering or lasting harm equivalent to, or higher than that caused by the introduction of a needle in accordance with good veterinary practice (for example hair loss, swelling under skin, body deformations). For any new mouse line generated or received, welfare assessment with increased frequency of observation and monitoring will be performed to ensure that the alteration does not harmfully impact the animals.

Animals undergoing surgical embryo transfer will experience short-lived post-operative pain and discomfort. This procedure will be completed under general anaesthesia so that the animal will remain unconscious, and in aseptic conditions, to reduce risk of infection. To minimise any pain or distress a range of available analgesia methods will be used. Animals undergoing non-surgical embryo transfer will experience no more than mild transient discomfort and no lasting harm.

Male mice undergoing surgery for vasectomy (i.e. male sterilisation, a surgical procedure to cut or seal the tubes that carry sperm to permanently prevent pregnancy) will experience short-lived post-operative pain and discomfort. This procedure will be also completed under general anaesthesia so that the animal will remain unconscious, and in aseptic conditions, to reduce risk of infection. To minimise any pain or distress a range of available analgesia methods will be used.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severity: Hormone injections

Breeding and Maintenance of Genetically Altered Animals

Embryo Transfer

Vasectomy (male sterilization procedure)

Mild 100% 100% 80% 0%

Moderate0% 0% 20%* 100%

Severe 0% 0% 0% 0%

*Moderate severity will be experienced by animals undergoing surgical embryo transfer. Every effort will be made to maximize number of non-surgical embryo transfers, which will result in mild severity.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to understand mammalian development, from fertilisation of the egg through to implantation in the womb and formation of the body up to day 10 post-fertilization. Many features of development are shared between mice and humans, including gene changes, embryo shape, cell identities and failures. Due to the significant ethical considerations of using human tissue for research, as well as the limited access to this resource, an alternative model is required. Mouse embryos represent the gold standard of mammalian development research as they resemble the human embryo in several key criteria. It has been shown that similar (homologous) genes, gene networks and mechanisms play role in early developmental of the mouse and human embryo.

Mouse embryos are also readily genetically manipulable, allowing for investigation of specific gene and cell functions in a way that cannot be done in the human embryo mainly due to ethical constraints.

Special facilities, designed to keep and breed mice in the most ethical conditions are also present and will be used in this project.

Development at these stages involves the interaction of different cell types, including the embryo itself, the placenta, the yolk sac and the maternal womb. Simple model systems, including stem cells (i.e. cultures of cells with the ability to develop into many cell types of the body) and/or embryo-like organoids (three-dimensional tissues derived from stem cells) do not capture this complexity and, therefore, are unsuitable for the research outlined in this project. However, it should be noted that our group seeks, wherever possible, to answer questions in a system not requiring the use of animals.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of multiple non-animal alternatives:

1. Stem cell-derived embryo-like models (i.e. tissues based on structures derived from aggregates of different stem cell types) have been successfully developed by our group and others. These structures can partially replace or complement experiments utilising natural embryos when studying some developmental processes.
2. 2D stem cell cultures are widely used in our laboratory, not only for generating embryo-like structures but also as a preliminary model to study selected phenomena occurring during early mouse development.
3. Computer modelling to understand early development. Computational models can be used to make and validate experimental predictions without the use of animals.

Why were they not suitable?

1. Stem cell-derived embryo-like structures are being used to complement our research on mouse embryos. However, these embryo-like structures do not undergo normal development (i.e. do not develop from a single fertilised cell), do not contain all of the correct cell types, arrangement or genes of the natural embryo. Additionally, extraembryonic tissues that are also indispensable for proper embryo development (e.g. yolk sac) are not fully developed and functional according to our current knowledge.
2. Simple stem cell models, including 2D cultures, are useful to probe very specific questions (e.g. gene function in one cell type) but do not capture the complex, many cell type-nature of the natural embryo. Indeed, we are particularly interested in the interaction between the embryo and the surrounding tissues (placenta and yolk sac), which cannot be modelled in 2D stem cell cultures.
3. Computer modelling currently does not capture the complexity of the embryo, including how the cell types signal to each other and change their gene expression. This data can only be generated from further experiments in the embryo.

The above stem cell and computer models are routinely used and improved to complement our animal research. Those alternatives already partially replace or complement experiments utilizing natural embryos when studying some developmental aspects. However, as mentioned above, due to complexity of the natural embryo

development and the changes in interactions between cells, which are not yet fully deciphered, it is still impossible to replace natural embryos in most cases.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The project application and research plans have been broken down to 4 separate aims. We have divided each aim into the questions we would like to answer, and the anticipated number of animals needed to get this data. With our long-term experience in breeding and maintenance of mice and embryo generation, recovery and culture, we can estimate how many mice we would need. This number takes into our project aims, as well as, our specific protocols (for example breeding and maintenance of genetically altered animals).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During experimental design we use all available published data as well as our unpublished data to ensure that the questions are valid, novel and addressed in the best possible way, based on the current knowledge.

When designing experiments, we are considering different scenarios and outcomes and potential for the future directions. Number of embryos and mice needed to obtain a significant result is revised for each experiment. Subsequent planned experiments are always revised with the most up-to-date results. The experimental plans are modified as necessary. This is continually discussed between the licence holder and group members. We also run pilot studies to reduce the number of animals and embryos used when we start a new series of experiments. This is done to validate experimental design and/or new protocols that we plan to implement, and to ensure that the results obtained are measurable and specific, allowing us to draw conclusions in the future.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Methods for mice breeding and maintenance, mouse embryo production, recovery and culture are well known and optimised to ensure refinement and reduction in animal use. Our group and animal units have long term experience in successful mating, regularly obtaining high yields of embryos. This is assured by maintaining the wellbeing of the animals (for example, through proper handling, reduction of stress and diet) as well by implementing good practices that increase the efficiency of successful mating and

superovulation. For example, there is maximum frequency of use for the males for the mating, mating is done in a 1:1 manner, males are continuously monitored, hormone injections are performed reproducibly (i.e. at the same time of day). This approach has reduced the number of failed matings (i.e. resulting in no embryos after superovulation, low embryo yields or bad quality embryos).

Embryos are placed into experimental groups randomly. This ensures experimental and control groups are similar and increases experimental success.

Additionally, we use reliable reagents from globally recognized manufacturers and suppliers. This approach decreases the number of failed experiments, ensuring that the methods used are highly efficient and reliable.

As a good practice, close oversight of the group and experiments by the Project Licence Holder is always assured. This is carried out across the breadth of the project, including by regular meetings (at least once a week) to discuss the progress of the project and future experimental plans, ensuring that the experiments are planned well and they approach the problem in the most efficient way, and by regular meetings with mouse line managers (again once a week) to discuss status, health, availability and performance of each mouse colony. Together this ensures that the mice are always bred and maintained according to best practices and with the reduction, refinement and replacement principals in mind.

Before proceeding to the actual experiment, we use available datasets whenever possible. For example, gene expression datasets, protein interaction databases and protein and gene features/functions databases. This preliminary step enables us to have additional insights regarding our experimental plans and questions and helps us to avoid redundant experiments.

We also run pilot studies to reduce number of animals and embryos used when we start a new series of experiments. This is done to validate experimental design and new approaches that we plan to implement, and to ensure that the results obtained are measurable and specific, allowing us to draw conclusions in the future. Finally, we also use computer modelling in several of our projects that use both mouse embryos and stem cells.

If there is a need coming from other groups, we are open to share animal tissues, including tissues from genetically modified mouse lines and post-mortem tissues, in order to further reduce overall mouse numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our experimental output are mouse embryos in different stages of the development. They are recovered from female mice mated with male mice. To obtain embryos for our research we use wild-type mice and genetically altered mice.

- embryo production protocol is a well-established and validated protocol. It brings only minimal suffering (i.e. mild severity) and is not anticipated to produce any adverse effects, beyond transient discomfort due to the injections, which will be intraperitoneal (i.e. into the body cavity) or subcutaneous (i.e under the skin). Transient discomfort results from restraining the mouse for the procedure for a short time (around 10-15 seconds) as well as from the injection needle.
- genetically altered mice bred and maintained for the project are not expected to experience harm due to the genetic alteration. All procedures related to the breeding and maintenance are expected to have a maximum of mild severity (and below that threshold in most cases).
- the only method currently available for allowing genetically altered embryos to develop is to implant them into the uterus of a pseudo-pregnant mouse. Thus, we cannot avoid this procedure. However, non-surgical embryo transfer methods will be used whenever possible as more refined method, where the success rate matches that of surgical embryo transfer methods or is satisfactory. However, in cases where it is not possible (e.g. transfer of embryos at very early stages, repetitive problems with non- surgical transfer efficiency that would not result in positive outcome), surgical transfer will be used.

Protocol for generation of genetically altered animals follows current standards and well-established procedures.

- we plan to use mainly genetically sterile males as an alternative to vasectomised males in generating pseudo-pregnant females.

Why can't you use animals that are less sentient?

Non-mammalian species (including Xenopus or Zebrafish) are often utilised in developmental studies and do share commonalities to both mouse and human development. However, these species diverge importantly in their developmental timing, gene expression and embryo shape in comparison to the human and therefore, are often inadequate models with which to draw conclusions on human development. The mouse embryo offers similarity to human development, while offering potential benefits.

To generate embryos, adult mice of reproductive age must be used. However, where possible, embryos are taken at the earliest point (from fertilization to day 10 post-fertilization when body plan is formed) to minimise life stage/sentience.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our group and animal facilities staff have long-term experience in maintaining wellbeing of the animals and consistently implement new training and protocols. High standards in the facilities are maintained to ensure the animals are less stressed and well cared for (e.g. by provision of an enriched environment, acclimatisation when animals are moved into the facility, placement of animals into social groups). By implementing good practices, we also increase well-being of the animals and, therefore, mating efficiency (which leads to reduction in use). For example, frequency of use for the males is limited, mating is done in a 1:1 manner, males' efficiency and behaviour are monitored, superovulation is performed in a repetitive scheme (hormones administered at a repetitive time of day). This approach reduces the number of failed matings, in turn reducing the number of animals required.

Establishment staff also have long-term experience in monitoring animal welfare and they ensure that every person involved in animal management in any way, must be first trained to the highest standards. If needed, increased monitoring and/or adequate pain management are implemented. Pain management will be achieved through the use of medicated palatable substances for voluntary treatment such as flavoured jelly, paste or milk shake liquid. We will get the animals used to the taste profile of the substances prior to the surgery so they will self-administer effective analgesia.

Humane endpoints are strictly adhered to minimise animal suffering during the project.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the guiding principles on good practice for Animal Welfare. We adhere to the new ARRIVE guidelines (2.0), where possible. We also attain to the Lasa guidelines to ensure good practice on animal work, as well as the NC3Rs published strategy for improving animal welfare (see publication details below).

- Prescott MJ, Lidster K (2017) Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Animal 46(4):152-156. doi:10.1038/laban.1217
- LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery. https://www.lasa.co.uk/current_publications/

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are regularly updated about advances in 3Rs by our Establishment staff (e.g. by Named Animal Care & Welfare Officers).

Additionally, we refer to available resources such as NC3R's (National Centre for the Replacement, Refinement & Reduction of Animals in Research) website page and 3Rs guidelines (https://nc3rs.org.uk/resource-hubs), LASA (Laboratory Animal Science Association) website page (https://www.lasa.co.uk/), RSPCA (Royal Society for the Prevention of Cruelty to Animals) website page (https://www.rspca.org.uk/), Norecopa (https://norecopa.no/databases-guidelines) and The Jackson Laboratory resources (https://resources.jax.org/).

We also take an active role in advancement of the 3Rs – for example we pioneered development of 3D stem cell models for mouse embryo development, which has been recognised by the NC3Rs (winner of the 2019 3Rs Prize).

78. Tumour suppression and therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Cardiotoxicity, Immunotherapy, Inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand mechanisms of tumour suppression, and to investigate potential methods for tumour therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a disease caused by genetic alterations or exposure to environment factors such as carcinogens, infectious agents or/and immune suppression. It has also been

considered as a disease of ageing, as the longer someone has been alive, the greater the chance of acquiring the changes that lead to cancer. Tumours arise following genetic changes that cause normal controls that regulate cell numbers to be overcome. Our understanding of these genetic changes has improved greatly over the past 30 years, but the changes that occur during cancer development are complex, and research continues so we can fully understand the nature of the genetic changes that arise in cells and how these changes ultimately result in tumour growth.

One aspect of our research is to analyse the causative mechanisms of cancers with low survival rates such as cancers of the oesophagus and pancreas. The 10-year survival rates for cancer of the stomach and oesophagus is around 12%, and for cancer of the pancreas it is as low as 5%. These two cancer types are among the 20% of cancers associated with chronic inflammation caused by lifestyle factors, and we intend to use mice to mimic these diseases with the intention of both gaining a better insight into these cancers and developing new therapies to alleviate these conditions. One consequence of a better understanding tumour development has been the advent of new treatments such as therapies that stimulate the immune system to destroy the tumour. Although there have been many successes with these therapies, the potential exists for novel applications, for example the combination of these therapies with other existing drugs to develop new treatment strategies.

The advancements in cancer therapy in recent decades has resulted in an increased life expectancy for many who receive a cancer diagnosis. As a consequence, the importance of long-term side effects of cancer therapies has become apparent. In particular, the toxic legacy of cancer therapeutics and radiotherapy upon the heart is of increasing concern, and we intend to explore the mechanisms whereby chemotherapeutics cause damage to the cardiovascular system.

What outputs do you think you will see at the end of this project?

We hope to increase our knowledge of tumour development and suppression mechanisms and use this to identify possible avenues for the development of potential novel tumour therapeutic strategies. We also aim to improve our understanding of the impacts of cancer therapies on the heart. The output will principally be the publication of our findings in peer reviewed journals.

Who or what will benefit from these outputs, and how?

The initial beneficiaries of this work will be other scientists working in the field of cancer research. Ultimately, our aim is to develop effective therapies that are of clinical benefit to cancer patients. We mainly intend to use drugs that are already used clinically either for novel purposes or in new combinations with other therapies. The starting point for deciding on the use of these novel treatments is ongoing in vitro work, which will be used to identify which agents (usually only one or two) to take forward into the mouse. The experimental

data using mice should be generated within this licence (3-4 years) but any clinical trials prompted by our work is beyond the scope and time frame of this licence.

Another aspect of our work involves some human genetic disorders, so we hope ultimately that our observations may contribute evidence towards expanding genetic testing of families with these conditions, both by determining the source of their inherited condition and the identification of potential carriers. However the final decisions on whether our work is of use in the clinic are beyond our authority.

How will you look to maximise the outputs of this work?

We intend to continue to publish our data in peer reviewed journals and present it at conferences and invited seminars. Our publications are announced on our organisation's UK and international websites.

Species and numbers of animals expected to be used

- Mice: 35750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are widely used to study development and disease, their biology and genetics are well documented and characterised. They have a relatively short life cycle, which reduces the time taken to generate data, and many reagents and experimental methodologies for use with mice are readily available. The technology to genetically alter mice is well-developed and reliable. This technology can introduce genetic alterations to the mouse to, for example, prevent the function of a particular gene, and comparison of the behaviour of a genetically altered mouse with a mouse that has not been mutated can tell us important information about the gene in question. Congenital birth defects and cancer in humans are both caused by errors in DNA, and mice can also be generated to introduce DNA mutations that copy these disease-derived mutations so that their consequences can be further investigated.

One of the major risk factors for cancer is age, so although much of our work will be undertaken using healthy young adults, some of the work will need to use aged mice (mice over 15 months) as some differences in tumour susceptibility do not become apparent until after this age.

Typically, what will be done to an animal used in your project?

Mice will be bred with genetic alterations that in some instances may mimic human disorders. These include mutations that affect the heart, which are thought to be related to

a group of inherited human heart conditions, and mutations affecting the central nervous system, which again may be related to genetic conditions causing abnormalities in humans such as epilepsy and intellectual disability. These mice will be investigated to provide more information about how these mutations cause their associated human conditions, for example by measuring the efficiency of their hearts by techniques such as MRI or ECG, or by microscopic or biochemical analysis of tissues collected post-mortem. Other mice will be bred that have mutations that are found in human cancers. Some of these mutations alone will cause tumours to grow in the mice, whereas others will predispose to tumour growth if further procedures are carried out. We will also make use of genetic technology that allows the mouse to develop normally, but enables the genetic alteration to be activated either at a specific time in the life of the mouse and/or in a specific organ or tissue by dosing the mice with the drug tamoxifen.

In this project, we aim to both examine how tumours grow and also how these tumours can be treated. Tumour growth may be caused by several methods. One method involves injecting tumour cells, either under the skin to produce subcutaneous tumours, or into the bloodstream to mimic the spread of cancer that causes secondary tumours. Another method is by exposure to cancer-causing chemicals or radiation, which may result in tumours in organs including the pancreas, colon or liver, or by infection with cancer-associated pathogens that can cause tumours in organs such as the stomach. The cancer causing agents will usually be injected into the peritoneum or may be given in the drinking water.

Irradiation of a mouse involves a brief exposure to X-rays.

Tumour type and rate of development will vary depending upon the genetic alterations of the animal and the cancer-inducing stimulus used. For some methods the experiments will only last a few months. However, cancer is a disease of old age, so for other methods the mice may need to be kept until they are over a year in age.

Tumour development will be measured by callipers if the tumour is external. In some experiments it may be possible to fluorescently label the tumour cells so that their movement can be tracked within the body using non-invasive imaging equipment, or use methods such as MRI to check for internal tumour growth. However tumour development will mainly be monitored by at least daily observation of the mice to check for signs that they may have developed a tumour. Limits are in place in this licence to preclude the growth of tumours such that they cause unnecessary suffering.

In some cases the mice will receive potential tumour therapies, which may include drugs or immune cells, to determine whether they affect tumour growth. Before these potential therapies are given to tumour-bearing mice an appropriate dose will be set using small pilot studies. In some experiments we will give some animals chemotherapeutic agents and measure the effect they have on the heart using standard techniques such as MRI or ECG.

All mice used in this work will ultimately be killed by a humane method and after the mouse has been killed tissue samples will often be kept for laboratory analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The study of tumour biology in mice is not possible without causing some animals to develop tumours. Tumours, depending upon their location and the method of induction, may cause weight loss, laboured breathing and pain, which will usually be evident by general malaise, social withdrawal and lethargy. Regular monitoring of susceptible animals will keep any such suffering to a minimum, and where appropriate the tumour size will be measured using callipers or where available using non-invasive imaging methods.

The administration of substances will frequently be by injection, which will cause mild and transient pain. Another route is by oral gavage which, when performed competently, should cause little discomfort. To determine an effective dose of the substance under test may require a small number of animals in pilot studies to experience toxicity at the upper dose which may include weight loss, and malaise similar to that demonstrated by the tumour bearing mice.

Some of the mice we use have a cardiac abnormality which causes progressive heart failure. These mice are usually in apparent good health until around 3-6 months of age, when the effects of the heart abnormality start to become apparent. This is observed by laboured respiration (panting) and reduced activity. Once a mouse is showing these signs, it will be killed by a humane method.

In addition to developing tumours, ageing mice may also display general signs such as ulcerated dermatitis, dental abnormalities, cataracts and general decline associated with a reduction in organ function.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most of the mice on this licence (>50%) are only involved in breeding and carry harmless genetic alterations. When breeding genetically altered mice, it is inevitable that mice will be generated whose genotype is not experimentally useful e.g. heterozygous mice who only have one copy of the mutant gene rather than two. These mice generally suffer no adverse effects from their genetic alteration and most are not used experimentally, although some are used in breeding.

Some mice will experience a moderate severity solely as a consequence of their genetic alteration. These will constitute up to 22% of the mice bred on this licence.

Of the mice that undergo experimental procedures, 80% will experience a moderate severity. This accounts for 29% of the total number of mice on this licence.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Living organisms are intricate combinations of numerous interacting tissue types, many of which are not fully understood. Tumour development reflects this, as it is influenced by multiple factors such as the immune system, blood vessels and surrounding tissues. This cannot be replicated in vitro. For some of our work we are using cell culture techniques such as organoid culture, which grows small samples of tissue from human patients in an artificial matrix to replicate in vitro some aspects of in vivo biology, and while we do derive useful data from this method, the technology is still in its infancy and cannot yet replace the use of animals.

Part of our work involves the immune and cardiovascular systems. Although some of the genes and signalling pathways that we study are conserved in nematodes and fruit flies, these organisms lack an immune system and cardiovasculature comparable to that of humans. Mice are the lowest mammals suitable for investigating tumour growth and the interaction of this with the heart and immune system, and their genetics and anatomy are often comparable to humans.

Which non-animal alternatives did you consider for use in this project?

We have gained valuable data regarding the biochemistry and cell biology of tumour suppressors, tumour development and treatments using cell culture techniques, and all our initial experiments are performed in vitro. In our laboratory we are also developing methods using organoids, which are three- dimensional multicellular cultures derived from human patient samples that can replicate in vitro some of the spatial organisation and function of their tissue of origin. These are useful for analyses of e.g. the gastrointestinal tract and its interaction with inflammatory and tumourigenic pathogens. This technology can be used to investigate the signalling pathways that are activated in these situations.

Why were they not suitable?

Organoid culture systems lack the surrounding microenvironment found in vivo, and the use of co- culture methods using combinations of different cell types in an attempt to overcome this is not firmly established. Similarly, when using potential tumour therapies, it is not possible to account for the in vivo pharmacokinetics of the drug when using organoid

culture. Organoid culture also works better on some tissues than others: for example, organoid culture using tissue from glandular tissues such as the intestine works much better than for stratified tissues such as the skin. Also, if human patient samples are to be used as the starting material for the organoid culture, sourcing these can be inconsistent and unpredictable.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on our past experience of the methods used in this licence and the breeding that was required to achieve the numbers of mice required for each study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Following consultation with statisticians, we have found that for tumour studies, around ten mice of each sex for both control and treated groups is sufficient for statistical significance. Survival curves are calculated using the log rank test with significance of $p<0.05$, and standard statistical tests such as ANOVA and t-test are used to analyse the data. This will continue to be reviewed and resources such as the NC3Rs Experimental Design Assistant will be consulted where necessary.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will follow the breeding guidance provided by our institution to optimise breeding performance, and strains of mice that are no longer required will be cryopreserved. The mice used in this work have been backcrossed onto defined background strains (e.g. C57BL/6, Balb/c) to reduce experimental variation.

Pilot studies using small numbers of mice will be undertaken, for example when using novel substances, to determine whether the experimental approach is feasible and appropriate.

Where appropriate, control cohorts may be used as reference in multiple experiments.

Similarly, for studies where tissues are harvested post-mortem e.g. paraffin-embedded tissues or frozen tissues kept for biochemical analysis, where appropriate these will be shared or used in multiple experiments or analyses.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This work will only use mice. Mice are the lowest mammalian species whose anatomy, physiology and genetics are suitable for research into human disease and are the most accurate animal model available for this purpose. Techniques for the genetic manipulation of mice are well established and many mouse models are available for use in our work.

Many of the genetic alterations in the mice we will use are harmless. Where possible we will confine any disease-related genetic alterations to the organ or tissue of interest, so that most of the mouse is functionally normal. Sometimes these genetic alterations will cause disease states like tumour growth, but we will avoid this where possible. We will sometimes allow the mice to age, which reflects the increased risk to health that comes with old age in humans, and some of these mice will experience a physiological decline as a result. For some of our work we will use mice with impaired immune systems. Although these mice can be subject to opportunistic infections, when housed under clean conditions they remain healthy.

We will use various methods of inducing tumour growth in mice. One such method is the introduction of human tumour cells into mice with impaired immune systems - this prevents the rejection of the human cells by the immune system of the mouse. This will enable measurement of the speed of tumour growth and is also the model that will be used to test potential novel tumour therapies. These cells are introduced under the skin so tumour growth is superficial and will not interfere with the internal organs. Therapies will, in many instances, be those already in clinical use. Any tumour therapies whose behaviour in the mouse is unknown will initially be tested in pilot experiments in small (2-3) groups of mice.

Many tumours in humans arise as a result of chronic inflammation, in particular tumours of the gastrointestinal tract and digestive system. In humans these conditions, e.g. pancreatitis, can often be painful and unfortunately this will sometimes be replicated in the mouse, although in the model used here the inflammation is transient and reversible. Other methods of inducing inflammation are minimally or non-invasive, and any adverse effects are reversible.

Some tumour therapies used in the clinic have unwanted side-effects on the heart. To investigate this, genetically altered mice will be given tumour therapies that are in clinical

use, and their heart function measured using methods such as MRI that are also used on patients. The agents will be given at the lowest dose possible. Some of the mice bred on this licence have cardiac abnormalities when they have two altered copies of our gene of interest. Although we may have to use a small number of these mice to collect information about the tumour therapies, the majority of this study will be carried out on mice with one altered copy of the gene that are less susceptible to heart complications. These mice should be better able to withstand the treatments they are given, and are more representative of the situation in the clinic.

Unfortunately when studying tumour biology it will be on occasion necessary for some mice to develop tumours. This will have a long latency, e.g. where the mice are aged, and the majority of the life of the mouse will be spent in good health. Other methods of inducing tumours, such as chemical carcinogens, are relatively quick, but again the mice will live most of their lives in good health. The radiation dose typically used in this licence to induce tumours has few if any short term effects. As this is a quick and non-invasive means of exposing the mouse to DNA damage, this will be used in preference to the chemical methods for an initial assessment of the tumour susceptibility of a novel genetically altered strain or cross.

Why can't you use animals that are less sentient?

Mice are the least sentient animals that have anatomy and physiology similar to that of humans. Although some of the some of the genes that we are interested in have equivalents in nematode worms and fruit flies, these less sentient animals lack the ability to grow tumours and not have sufficiently complex immune systems to be able to make meaningful comparisons with humans. The development of tumours and their interplay with the immune and cardiovascular systems are dynamic processes that requires a living animal. Age is an important contributory factor to tumour development, so the use of aged animals is required to accurately reflect this.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice are routinely housed in individually ventilated cages to minimise the risk of infection, and supplied with environmental enrichment.

When administering agents to the mice we will use the least invasive route possible, for example administration of viscous substances by gavage rather than injection.

Observed outcomes of the tumour studies carried out on this licence will be used to revise endpoints in future studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will make use of the resources available at the NC3Rs website (www.nc3rs.org.uk), including the NC3R's Experimental Design Assistant where relevant, and follow the ARRIVE guidelines (https://arriveguidelines.org) when undertaking and publishing this work.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All staff who carry out work under this licence attend local good practice and welfare meetings, and we are in receipt of regular e-mails from the Named Information Officer at our establishment.

Attendance at the local annual NC3Rs day will be encouraged. We will consult the NC3Rs website for the latest updates.

When undertaking a procedure for the first time, we will consult the Named Information Officer, vets and NACWO beforehand so that any recent 3Rs updates can be implemented.

79. The effects of aging and prior injury on tissue regeneration and fibrosis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Aging, Fibrosis, Senescence, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will explore the mechanisms by which aging and tissue injury impact on the ability of the body to repair fully after injury, and its propensity to develop harmful scarring. By understanding these mechanisms, we hope to be able to manipulate them to prevent organ fibrosis and promote complete repair after injury

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Organ fibrosis becomes more common with age and after injury – and accounts for up to 45% of deaths in the developed world. There is an urgent, unmet need for new therapies to prevent progressive fibrosis – and in turn this requires a better understanding of the underlying processes driving the accumulation of scarring with aging and after injury.

What outputs do you think you will see at the end of this project?

A key goal of this project is to identify factors accounting for the sensitivity of the aged kidney to acute kidney injury (AKI) and inadequate repair.

In the short term (within the first two years) the aim is to identify changes in the behaviour of cells and the expression of genes and circulating proteins (known as biomarkers) which may be associated with the development of AKI.

The aim is to then generate new therapeutic interventions after the end of this 5 year PPL to

limit AKI and promote renal recovery in the elderly,

Reduce mortality via the development of new treatments.

Reduce morbidity and cost to the NHS (and hence to the public).

Given the annual incidence of AKI, its high mortality and the increased risk of chronic kidney disease and premature cardiovascular death in survivors there is the potential to save lives and to significantly reduce the excess costs to the NHS associated with AKI diagnosis and treatment. The current costs for this were recently estimated at several hundred million pounds annually.

This study is designed to generate information for clinical translation, but will do this by increasing our understanding of the basic biological processes of aging and how they can be manipulated. By the end of the project we should understand more about the basic biology accounting for renal aging. We will investigate how aging impacts on the function of the immune system and whether the age of the injured organ contributes to the behaviour of immune cells which are present in the aftermath of injury

In addition, we will seek to identify proteins which promote scarring in aged kidneys. As renal fibrosis is a major pathological and prognosis feature of human kidney disease, strategies that retard progression or accelerate regression of fibrosis are likely to be beneficial.

Furthermore, as fibrosis is a common end-point shared by diseases of many organ systems, anti-fibrotic therapies are likely to have broad translational potential. The longer term goal will be to translate any positive findings to the clinic to minimise the numbers of patients progressing to end stage renal disease or failure of other organ systems. If successful, in the longer term (beyond the end of this PPL) this could generate major population health and economic benefits.

Who or what will benefit from these outputs, and how?

The stated objectives have common potential short and long-term benefits and losses if not achieved; Short-term benefits will be:

- A greater understanding of the cell biology of organ fibrosis and repair, especially with regard to the identification of the major cell populations promoting fibrogenesis and tissue regeneration. This will benefit scientists in the near term (years 1-2 of PPL) as this will allow us to focus on the major fibrosis- promoting subpopulations of cells within the kidney, allowing in depth characterisation of these key sub-populations, with the goal of developing novel treatments. We will disseminate our findings widely via publications / lectures / conferences.

- The development of effective inhibitors (small molecule or antibody based) that limit organ injury, accelerate tissue regeneration and limit fibrosis in animals. Taking the therapeutic targets identified in 1 above, we will then assess small molecule inhibitors/antibody-based therapeutic approaches to inhibit fibrosis production by this subpopulation of pro-fibrotic cells in pre- clinical models in vivo (years 2-3 of PPL). We will disseminate our findings widely via publications / lectures / conferences.

- Increasing our experience and further refining the use of cutting-edge imaging techniques (e.g. MRI, ultrasound, optical imaging, fluorescent imaging via abdominal imaging window [AIW]) in the diagnosis and monitoring of organ injury and fibrosis in preclinical models of kidney fibrosis will also reduce the numbers of animals used for biomedical research, which if adopted more widely in research institutes around the world will have a major impact on reducing the numbers of animals used in biomedical research. We will disseminate our findings widely via publications / lectures / conferences (years 1-5 of PPL).

Longer term benefits might be:

- The use of small molecule inhibitors or antibody-based therapies to modulate organ injury and regeneration in patients with organ injury and fibrosis, including the development of both anti-fibrotic and pro-regenerative medicines. There is a huge unmet clinical need in organ fibrosis, so if we can identify potent new anti-fibrotic therapies this could have a massive impact on human health.

- The effects on organ transplantation are potentially far reaching. Effective therapies for acute kidney injury and the subsequent fibrotic responses to this has the potential to both minimise new cases of progressive chronic kidney disease (CKD), and to extend the functional life of each kidney transplant performed. Additionally, developing treatments to retard the progression of organ fibrosis once injury has taken place could reduce the need for transplantation (and/or dialysis in the case of chronic kidney disease) and therefore reduce the burden of morbidity and mortality associated with these therapies.New treatments which can reduce or completely avoid the need for transplantation would be of benefit to the massive number of patients with chronic kidney disease worldwide.

- Identification of other therapeutic targets that may modulate kidney injury and scarring or accelerate renal epithelial regeneration, this may be clinically relevant for the treatment of patients undergoing procedures such as cardiac bypass grafting or aortic aneurysm repair which carry high risks of acute kidney injury.

- Identification of non-invasive biomarkers of fibrotic disease and response to therapy in fibrosis of the kidney. Successful identification of non-invasive biomarkers for fibrotic disease would reduce the need for invasive tests such as a kidney biopsy, which is currently the gold standard for diagnosis of human kidney fibrosis, but can result in significant morbidity and even mortality.

How will you look to maximise the outputs of this work?

Scientific Community

The work undertaken during this project licence will be relevant across diverse specialties and disciplines. We are committed to publishing our findings in journals with an open access policy; maximising access to our work across the wider scientific community: including basic scientific researchers in fields including ageing, senescence, bioinformatics and fibrosis, clinicians and pharmaceutical researchers. We will present at local, national and international meetings and we expect these will span diverse areas including nephrology, transplantation, aging and senescence. As a group we strongly believe in developing, maintaining and learning from the sharing of knowledge and information. A component of this fellowship will dissect senescent cell heterogeneity including significant amounts of RNA sequencing; the results of which will be will be uploaded to open access repositories.

Patients and public

Despite high incidence and serious morbidity and mortality, CKD does not have a prominent public profile. The work undertaken during this project licence focuses on an area of basic science with clear relevance to progressive fibrotic CKD, and we will seek opportunities where possible to involve ourselves in initiatives which might change this. We anticipate these including public lectures and fundraising functions organised by the MRC. Our unit has a strong tradition in public engagement and employs a Public Engagement and Communications officer. This staff member can assist with summarising published scientific papers in easily lay-accessible overviews, providing a direct, accessible voice (independent of external media interpretations) to open up our published research to interested adults, patients trying to find information about their diseases and new treatments, patient interest groups, funders, lay members on science-related Committees, and senior school groups conducting their curriculum section on current and local science research.

We are also keen to enlist their help to maximise opportunities to inform patients and the wider public about our scientific work. Opportunities include 1) Science Festival workshops and Drop-in events and School workshops, presentations, career and mentoring opportunities.

Charities

We believe that there is ample evidence implicating senescence as relevant to fibrotic disease processes in multiple organ systems. By publishing our results in open-access,

high impact journals, and liaising with the public as detailed above we will engage charities to fund further clinical studies to translate our work 'from bench to bedside'.

Government departments

CKD is a source of major morbidity, premature mortality and a major source of NHS expenditure in the UK (estimated as 1.3% of total NHS budget in 2010). It is our goal via the publication and engagement plans above that results generated by this PPL will reach a broad audience, and in the medium to long term lead to clinical trials (funded by government departments, industry or medical charities) and novel therapies to reduce patient morbidity, mortality and NHS expenditure.

Nephrology is now well represented in social media; often with hugely valuable outcomes and, where appropriate and of value, we expect to be active in this respect as well.

Species and numbers of animals expected to be used

- Mice: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Experiments will all be undertaken in adult mice, aged up to 2 years old.

The mouse has been selected for these experiments due to its short lifespan (aiding the study of all stages of the life cycle), its comparable mammalian renal anatomy, and the wide availability of transgenically modified animals allowing refined experiments to be designed to probe the importance of specific genes in the initiation and progression of renal fibrosis.

We will hold animals for up to 2 years, as aging is a well recognised risk factor in humans for kidney fibrosis. By studying animals aged 18-24 months old and comparing these to young animals, we maximise the chances of identifying physiological changes predisposing to fibrosis which will be relevant to human disease.

Typically, what will be done to an animal used in your project?

A typical animal used in this project will be bred in house under protocol 1.

At some point between the age of 8 weeks and 24 months of age, the animal will transfer onto another experimental protocol and undergo baseline observations, optionally including a small volume blood sample via the tail vein, and optionally including a 6 hour timed urine collection to allow detailed measurement of baseline kidney function.

The animal will then undergo a procedure to induce kidney injury, such as unilateral renal ischemia – followed by close post-operative monitoring, daily weights and analgesia.

The animals will be allowed to recover and followed for typically up to 10 weeks post surgery, with optional blood and urine collection within Establishment Guidelines, prior to cull under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Overall adverse effects

- Pain. Mild severity pain may result from ear notch sampling to permit identification and genotyping of transgenic animals, however this should be transient and no healing problems would be expected. Several of the surgical and non-surgical models used to explore the impact of various interventions on the duration and recovery of acute renal failure may cause wound pain, which whilst often only mild, may be of moderate severity. This will be actively looked for, and medication in the form of painkillers will be given to reduce its effects on the animals. Duration would generally be 48 hours or less.

5. Weight Loss. Animals may experience weight loss in the aftermath of a surgical or non-surgical intervention. This will be actively looked for, and animals will be treated with painkillers to minimise the impact of pain on food and drink intake. From our experience with these techniques, any weight loss has ceased by 48 hours post intervention, and any animal approaching 10% weight loss will be reviewed and will be culled to ensure their weight loss does not exceed permitted levels.

6. Malaise due to symptomatic renal impairment. We have designed our experiments to minimise the chances of animals experiencing symptomatic renal failure. In a small proportion (<5%) of the minority of animals undergoing a renal injury to both kidneys, the animals may become symptomatic, with hunching, reduced activity and weight loss. Should any of these symptoms be thought to be present the animal in question will be reviewed promptly and if indicated by their condition, culled to prevent severity limits being breached.

7. Transient sickness and diarrhoea. Sickness and diarrhoea can be a symptom of animals undergoing total body irradiation (TBI). This should be mild in nature, and will be controlled by regular inspection and if there are signs or symptoms such as weight loss of 20% of pre TBI weight, persistent diarrhoea, reluctance to move or feed (persisting despite adequate analgesia being administered for 48h); animals will be humanely killed. Antibiotics will be used according to the advice of the named veterinary surgeon. Animals will be housed in individually vented cages for 8 weeks post TBI to reduce the potential for infections but if infections or other health problems occur, the named veterinary surgeon will be consulted, and if possible measures will be taken to modify the intervention or make other changes that will minimise the risk of the problem recurring before the experiment is repeated. Animals showing signs of continuing disease or distress (as described above) will be humanely killed. Long- term effects are not expected in this protocol.

8. Complications of aging. Animals of greater than one year of age will be subject to additional weekly review to ensure there are no signs of unexpected weight loss,

hunching, piloerection, or altered behaviours indicative of mouse illness. Any animals exhibiting moderate or sustained features of illness such as unexpected weight loss of >10%, hunching, piloerection, or altered behaviours persisting for 48hrs will be humanely culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse - Protocols are either (mild) or (moderate) - with approximately 50% expected to be (mild) and 50% (moderate)

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Kidney failure is a complex and poorly understood disease encompassing both systemic effects and injury to multiple cell types. We will use carefully selected in vitro assays of renal cell behaviour to add value to the animal studies performed, and to minimise the need for in vivo experimentation as much as possible, but there is no alternative approach which is currently capable of replacing in vivo studies.

Which non-animal alternatives did you consider for use in this project?

We considered the use of computer based simulation in place of in vivo work, but determined that no suitable computer simulation exists for the studies planned. We will use in vitro studies in place of in vivo work whenever feasible - such as testing the behaviour of senescent renal epithelial cells using in vitro culture conditions to minimise animal use.

Why were they not suitable?

No computer based simulation is available which adequately recapitulates the function of the injured and/or aging mammalian kidney.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based the numbers above on our extensive experience in the chosen disease models, the anticipated group sizes and timepoints, and the necessary levels of cross-breeding and maintainance of the transgenic strains required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

At all stages we have planned our experiments to ensure that we are using the minimum numbers of mice necessary to answer our experimental questions, using tools including nQuery and NC3R's Experimental Design Assistant. Where possible, we will use refinements to allow multiple readings to be obtained from a single animal rather than multiple experimental repetitions, leading to less overall animal use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our extensive published experience in this field allows us to design experiments using the minimal number of animals necessary to answer the experimental question. Rational breeding strategies will be employed to ensure that the maximum number of animals are usable in experiments to minimise waste. We stay abreast of the latest research in the field, and will be open to sharing our own tissue stocks, or using existing stocks from other researchers to prevent unnecessary duplication of any in vivo work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used in light of the ready availability of genetically modified mice available for study of disease pathways and processes.

We have selected surgical and non-surgical models of acute kidney injury and progressive kidney fibrosis that equate to human disease. We will use a short-term kidney blood flow restriction model (IRI) and a urine flow restriction model (UUO) both of which are good models for acute kidney injury in people. We are familiar with these models and have refined them to use the minimum required kidney damage by reducing the time of blood flow restriction, limiting the degree of suffering experienced by the animals. As an example, in the large majority of experiments only one kidney will be injured, removing the risk of the animal developing symptomatic renal failure, due to the presence of a functioning kidney. We will always seek veterinary input in the event of any concerns about the condition of mice under this protocol, to ensure that signs of distress are not missed. Pain killers are always administered before and after surgery to limit discomfort experienced by the mice. Humane endpoints will always be implemented for all experiments. Additional models including nephrotoxin induced renal injury allow induction of renal injury without any surgery – again with the goal of minimising post-surgical pain and distress to the animals. The use of the newly developed model of heterochronic blood exchange allows the effects of mixed old and young serum on kidney injury, fibrosis and repair to be assessed for the first time without a major, permanent skin connection being formed between two mice – a significant refinement over conventional heterochronic parabiosis.

Additionally, this will often permit both paired mice to be used in the subsequent analysis of the experiment, which halves the numbers of animals required compared to conventional parabiosis surgery.

Why can't you use animals that are less sentient?

There are no less sentient animals available with a mammalian kidney. Due to the periods of time (weeks) required for overt and quantifiable fibrosis to develop after injury performing the entire experiment under terminal anaesthesia is not possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will be proactive in implementing any new monitoring and welfare refinements as they become available. Our goal is to maximise the research impact of each mouse whilst minimising the harm associated with this. As part of this, we ensure that all animals undergoing procedures receive heat pad support followed by overnight housing in a warmed box to minimise risks of hypothermia. Food softened with water is always placed on the flooring of cages to ensure it is easy for all animals to access fluids and nutrition. We have comprehensive in house protocols to ensure animal weights, activity and condition are documented pre/post all procedures. In the course of the last 5 years we have increased our use of models where only one of two kidneys is injured, with the goal of minimising the risk of symptomatic renal failure as a potential side effect of our kidney injury studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will make use of the ARRIVE guidelines from the NC3Rs to maximise the quality and reliability of our research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend institutional and national events and webinars organised by the National Centre for the Replacement, Refinement and Reduction of Animals in Research – and the project licence holder already subscribes to the NC3Rs newsletter. Any advances relevant to our project will be implemented via revision during the period of this PPL.

80. Zebrafish models to identify determinants of outcome in immune-mediated disease

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Tuberculosis, Immune response, Zebrafish, Inflammation

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve current understanding of protective and damaging effects caused by our immune system's response during tuberculosis (TB) infection and other immune-mediated diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tuberculosis (TB) is caused by mycobacterial infection. Knowing how to induce a beneficial immune response to this infection that does not injure the body's tissues would allow us to design better treatments for TB and new vaccines that induce effective immune defences so that TB disease never develops. In a broader context, understanding how to harness the immune system to control noxious agents without detrimental consequences offers opportunities to develop new and more effective therapies for diverse inflammatory and immune-mediated conditions.

What outputs do you think you will see at the end of this project?

This project will generate new information on protective and damaging effects of the immune system during tuberculosis and other inflammatory conditions, which I will publish in scientific journals.

Who or what will benefit from these outputs, and how?

By the end of this project, I hope to gain new insights that help to develop novel interventions that will significantly shorten the length of anti-TB treatment, which will be important to reduce spread of TB and minimise development of drug resistance. I anticipate that in the longer term, this work may also lead to design of new vaccines that prevent TB disease altogether.

New knowledge generated as a result of this work may also be applicable more widely, to aid design of new therapeutic strategies across a range of immune-mediated diseases for which current treatment options are limited, such as coronavirus disease 2019 (COVID-19) and other inflammatory and scarring lung diseases.

How will you look to maximise the outputs of this work?

I will publish my findings in scientific journals and make them available, where possible, before formal scientific review via public repositories such as bioRxiv. I will highlight new knowledge using Twitter and by presenting my data at scientific conferences and in seminar programmes at my own institution and those of my collaborator's institutions. Gene expression data from the work will be made available via suitable platforms such as Gene Expression Omnibus (GEO) and ArrayExpress, to allow other interested investigators to access this information for the purposes of their own research.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The zebrafish Mycobacterium marinum infection model leads to a type of inflammation that is an excellent model for human TB. The zebrafish is an accurate model of the mammalian immune system, in which levels of genes that influence the immune response can be genetically manipulated to assess their function. In addition, immature (larval) zebrafish are transparent, allowing bacterial growth and cellular responses to infection or injury to be viewed.

Adult zebrafish will be used to generate zebrafish larvae required for experiments but all experiments will be performed on larvae less than 5 days post-fertilisation (dpf), which are not considered protected by the Animals (Scientific Procedures) Act '1986'.

Typically, what will be done to an animal used in your project?

Adult zebrafish will be bred to maintain stocks required to generate embryos/larvae for experiments. These fish will live a normal, healthy life as any aquarium fish. A small biopsy of the tail fin will be taken from some fish in order to determine certain genetic characteristics, typically only once in an animal's life time.

What are the expected impacts and/or adverse effects for the animals during your project?

There are no expected adverse effects. However, rare adverse effects (less than 1%) may include abnormal swimming, infection and abnormal development or phenotypes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected level of severity is mild for all animals.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal use is necessary for the proposed work to study dynamic interactions between the immune system and inflammatory stimuli (TB bacteria or sterile injury) in a living host and to genetically modify components of the experimental system (knocking out or over-

expressing genes), which is not possible using human subjects or laboratory cell culture techniques.

Which non-animal alternatives did you consider for use in this project?

I considered using laboratory cell culture techniques to assess interactions between immune cells (macrophages) and bacteria or other immune stimuli.

Why were they not suitable?

These models cannot replicate the complexity of the immune system in a living organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This project continues the work conducted over a period of 5 years under my current project license and uses similar experimental designs. The estimated number of animals used for the proposed work is based on the numbers used during my current project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Genes will be screened for effects on immune responses by generating knockout zebrafish embryos using a novel CRISPR genome editing technique. All screening experiments will be performed on embryos before the onset of independent feeding, which are not considered protected by the Animals (Scientific Procedures) Act. Adult zebrafish carrying germline mutations for genes to be tested will only be generated where CRISPant (knockout embryos) show differences in immune responses.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals will be raised according to a protocol that has a high survival rate, reducing the number of animals generated overall. Sperm harvesting and freezing (cryopreservation) will help minimise the number of animals produced as unused lines can be archived and regenerated when required, with no need to continuously maintain lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project uses immature (larval) zebrafish models of inflammation. The zebrafish is an accurate model of the mammalian immune system, in which levels of factors that influence the immune response can be genetically manipulated to assess their function. In addition, larval zebrafish are transparent, allowing bacterial growth and cellular responses to infection or injury to be visualised. The zebrafish larval Mycobacterium marinum infection model provides a natural host-pathogen pairing which leads to inflammation that is an excellent model for human tuberculosis (TB).

All experiments will be performed on larvae before the onset of independent feeding; these larvae are not considered protected by the Animals (Scientific Procedures) Act. Adult zebrafish will be used to generate the larvae required for experimentation. These fish will live a normal, healthy life as any aquarium fish.

Why can't you use animals that are less sentient?

In choosing the zebrafish and focussing my experimental work on zebrafish larvae, I have selected the organism with the lowest neurophysiological sensitivity possible for work which evaluates the immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be raised according to a protocol that has a high survival rate, reducing the number of animals generated, as well as unnecessary suffering and fatality. Fish will be closely monitored by highly skilled staff to ensure that animals are bred at safe intervals and when it is beneficial to their health (preventing egg-bound females).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will follow guidance set out in the Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE) guidelines when planning my experiments and adhere to the standards set out by the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines when reporting my findings. In line with the ARRIVE guidelines, once experiments have been performed using a required fish line, sperm will be frozen and stocks will not be maintained further. Sperm harvesting and

storage minimises numbers of live fish generated/maintained but does require use of anaesthetic and handling, which may be stressors. However, current techniques do not require death of the fish, and are performed by highly skilled staff trained in these procedures, minimising stress to the animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep up to date with the latest developments in the 3Rs via the news feed from my institution's Biological Services Unit, monthly newsletters from the NC3Rs and the NC3Rs Twitter account. I will aim to incorporate any relevant advances within my work as soon as possible.

81. Antibody production for clinical and research applications

Project duration

5 years 0 months

Project purpose

- Basic research
  - Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Antibodies, Antigens, Peptides, Immunogens, Animals

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult
Rabbits	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The production of animal-derived antibodies to order, to be used to address multivarious biological and biomedical questions posed by the scientific (basic, preclinical and clinical/diagnostic) and pharma communities.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The production of high quality validated animal-derived antibodies are critical tools to study biological systems (in all kingdoms) and are vital to identify for example, new biomedical targets for therapeutic benefit. They are also essential for diagnostic kits for swift patient/animal diagnosis.

What outputs do you think you will see at the end of this project?

The output will be a product ie the animal-derived antibodies. Their production will contribute to novel and crucial basic science information to further our knowledge. The new knowledge will be published in the appropriate journals by the end user of the product. The new knowledge as well as the antibody can be used in preclinical and clinical settings to treat disease as well as be used as diagnostics.

Who or what will benefit from these outputs, and how?

The animal-derived antibodies will benefit the scientific community by purchase from commercial sources or through availability from the academic research community. The antibodies are critical tools to the biological/chemistry and biomedical sector for advancing basic research, as well as for therapeutic and diagnostic use.

How will you look to maximise the outputs of this work?

Outputs maximised by constant dialogue with the customer about immunising agent preparation for the best animal-derived antibody production outcome. Sharing and receiving best practice with relevant stake holders and contributing to researcher outputs and publicising the framework we work within.

Species and numbers of animals expected to be used

- Mice: 1100
- Rats: 200
- Guinea pigs: 150
- Rabbits: 2050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult rabbit, rat, mouse and guinea pig will be used. At this life stage the animals have reached maturity, a stage of development, that will contribute to a strong immunological response.

For maximial outcome success the end-user of the animal-derived antibody will need to use multiple unique target specified antibodies from a range of species and be able to identify them in their application (tissue/diagnostic test). Raising each unique target specified antibody in the same species for use in an individual application makes it impossible to then identify the antibody with a marker tag. The marker tag uses species as its recognition feature. Unique antibodies from the same species will be identified by the same marker tag making it impossible to differentiate between each antibody and specific target. Therefore, access to target specified antibodies from different species allows marker tags recognising each species and thus differentiation between each antibody and specific target.

Typically, what will be done to an animal used in your project?

For the production of an appropriate animal-derived antibody for multiple research purposes, the animals will receive an immunisation with the required immunising agent. After an appropriate interval (2-6 weeks following the primary immunisation) the animals receive "booster" immunisation of the immunising agent. Up to 18 booster immunisation may be administered again every 2-6 weeks.

Following this, to obtain polyclonal antibodies, the animal is terminally anaesthetised and bled by cardiac puncture, blood collected then humanley killed. Further processing (by client) is required to extract the relevant antibodies. For monoclonal antibodies, animals with a strong immune response will receive either an intrasplenic or intravenous immunisation with the immunising agent. Three- seven days later, again animals wil be terminally anaesthetised and bled by cardiac puncture, blood collected and the spleen may be removed. Following this the animal will be humanely killed. Further processing (by client) is required to extract the monoclonal antibodies.

What are the expected impacts and/or adverse effects for the animals during your project?

Immediately following immunisation no adverse effects are expected. However, transient discomfort may be felt at the immunisation site (similar to that experience after vaccination-eg Covid-19). If this is the case then pain relief can be adminsitered. Occassionally within the animal following the immunisation, as the immune response develops they may appear lethargic. These effects should be transient with resolution within a few days. At the immunisation site a "clump" of immune response cells (white blood cells in response to the procedure) called a granuloma may form. The "clump" will gradualy disappear within a week.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Monoclonal antibodies - Moderate -100%
- Mice 100%,
- Rats 100%,
- Guinea pigs 100%,
- Rabbits 100%
- Polyclonal antibodies- Mild -100%
- Mice 100%,
- Rats 100%,
- Guinea pigs 100%,
- Rabbits 100%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

ECVAM recommendations (May 2020 doi.org/10.2760/091625) urges an adoption of technologies for the generation of non-animal-derived antibodies if available and scientifically justified. This is currently aspirational.

Animals are still necessary for the generation of antibodies. For novel antibodies the ability to recapitulate as recombinant antibodies using universal genetic sequence libraries does not exist. Universal (non-animal-derived) antibody libraries contain an extensive array of diverse antibody genes, but you cannot use the library unless you know the sequence of the antibody. The antibody sequence cannot be determined from the immunising agent sequence alone. When the mammalian immune system is exposed to the immunising agent a myriad of diversification processes leads to specific B cell production. The B cells undergo further consolidation using the innate biological immune system to establish an antibody that is selective for binding affinity, specificity and protein stability. The immunising agent presented to a universal library cannot do this. The resultant animal-derived antibody can be sequenced then presented to the library and recombinant (non-animal-derived) antibody generated that is selective for binding affinity, specificity and protein stability. Only at this stage is the animal derived route unnecessary. This situation has not been arrived at.

Where a novel target (basic research/therapeutic/clinical) or an existing antibody source is near depletion/depleted and no antibody sequence is available then the antibody must be raised in an animal. Once the antibody has been produced it then must be sequenced and future production can then be derived from recombinant technologies.

We will not be progressing towards alternative ways to generate antibodies and thus replace the use of animals. We will refuse to use an animal if a non-animal alternative is available. We have in place a robust ethical process for accepting/rejecting requests with the final decision resting with the Animal Welfare Ethical Review Board (AWERB). Decision outcomes could be 1) Accept, 2) Request more information before decison or 3) Reject. AWERB will review annually the decision making process to ensure it is still fit for purpose to meet compliance with the Act and conditions of the licence. Our role is as as service provider and not a manufacturer of antibodies. Our ethical review process makes the client take responsibility to demonstrate non-animal alternatives are unsuitable/unavailable and where they are to move to non-animal-derived antibody production where applicable. We can help with this transition in the long-term.

Moving from animal to non-animal-derived antibodies requires a transition period. A complete move now risks halting our fundamental (basic research), clinical and therapeutic capabilities and removing an important research tool that benefits society in its widest context.

Which non-animal alternatives did you consider for use in this project?

We have not considered non-animal alternatives. We are a service provider and not a manufacturer of antibodies. It is the responsibility of the commercial antibody manufacturer to determine the suitibility of any non-animal alternatives (display and aptamer technologies, non-animal derived antibody libraries). If they are suitable we would not use an animal.

Why were they not suitable?

An expert body recommends adoption of technologies for the generation of non-animal-derived antibodies if available and scientifically justified. This is currently aspirational.

The purpose of antibody generation is to produce antibodies that recognise novel targets that allow fundamental, clinical and therapeutic activities that benefit society as a whole. Technologies to generate non-animal derived antibodies still fall short in achieving this when compared to animal- derived antibodies.

The technologies might be available, but utilisation as replacements cannot be fully realised until the molecular basis of the starting component (antibody protein sequence) is known and therefore now are scientifically unjustified.

In order to determine the antibody sequence, you need the antibody and the animal is that source. The antibody sequence cannot be determined from the immunising agent alone. Once the antibody sequence is determined then display technologies can be used to produce animal free reagents as an infinite resource. The science is not yet in place for full realisation.

Even where the novel antibody sequence is known the display technologies have problems recapitulating antibody structures especially if related to viruses. This is important in vaccine development because valuable detailed knowledge about how the animal immune system reacts to the immunising agent is determined. This crucial information is lost when using display technologies. Therefore, animal-derived-antibody for certain applications will always be the only viable means.

The antibody derived from a mammalian immune reaction replicates an almost infinite number of ways the immune system has adapted to the immunising agent. The resultant antibody has a much higher affinity and specificity for its target. The technologies at the moment cannot achieve this, so the resultant antibody does not function as well in its end application.

To improve the quality of the non-animal-derived antibodies requires substantial experimentation, experience, time and resources. This needs to be worked towards but in the interim animals are still central to antibody production.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on 10+ years experience on providing the service and the demand generated by external and internal customer requests.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The selection of an immunisation regime (volume/concentration) to initiate an appropriate immune reaction combined with the number of repeat immunisations enables production of a high quality animal-derived antibody . Application of this procedure in no more than 6 animals ensures an individual project can produce the maximum amount of antibody negating the need to repeat the protocol to produce more antibody in this way. Also, the regime enables the spleen to create and store the cells primed to produce the antibody. At the end of the project when collecting the antibody, the spleen is also removed and processed to give a cell culture that can produce the antibody indefinitely. Also, the antibody can have its protein sequence determined to go on to be produced by display technologies so future production would not require an animal.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The approach of immunisation (including boosters max 18) of between 2-6 animals with the same immunising agent using a volume/concentration that initiates an appropriate immune reaction provides a robust mechanism to achieve an optimal outcome. As each immunising agent is novel the standard approach has flexibility to prevent additional animals having to be used and achieve the best from the animals in project. For example, if the immunising agent is a small molecule these can be less successful at stimulating an immune response, taking longer to reach an acceptable antibody concentration within the standard protocol time frame. Options are available to avoid increased animal use including additional boosters with longer time interval between booster allowing the animal the maximum time to respond and/or double dose (increase antigen concentration in same starting volume) for the booster immunisations. It must be recognised as each immunising agent is unique it is hard to determine the factor that causes a low response. The standard protocol with flexibility ensures the best outcome for the minimum of animals used to avoid recruitment of additional animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The adult life stage for each species (rabbit, rat, mouse and guinea-pig) will be used because their immune system is considered to have reached maturity ensuring a complete biological response will be achieved when exposed to immunising agents.

The methodology comprises a series of injections of the immunising agent with experience of transient discomfort akin to a human /animal receiving a therapeutic injection eg Covid-19 vaccination.

Why can’t you use animals that are less sentient?

The adult life stage for each species (rabbit, rat, mouse and guinea-pig) will be used because their immune system is considered to have reached maturity ensuring a complete biological response will be achieved when exposed to the immunising agents.

Animal-derived antibody generation requires an interaction between the immunising agent and the immune system so a living system is required. Adult animals will allow the generation of large volumes of antibody.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All involved staff are already trained to a high standard to carry out the immunisation procedures, welfare and husbandry of the animals. New staff will receive training to maintain the high standards we work to. Good communication among individuals results in quick response and resolution to emerging issues. All these will be maintained. Continuation of the single use needle policy that was introduced in 2019.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3R -https://www.nc3rs.org.uk/experimental-design NC3R -https://nc3rs.org.uk/reuse-of-needles

NC3R -https://nc3rs.org.uk/grimacescales

LASA Good Practice Guidelines -
https://www.lasa.co.uk/PDF/AWERB_Guiding_Principles_2015_final.pdf

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Dialogue with colleagues and forum networks undertaking similar work. Engage with NC3R website and webinars (Moving to the Use of animal-free antibodies, July 2020).

Within the frame work of relevant advances in relation to the production of animal-derived antibodies, these will be tried alongside existing practices and compared to evolve the best approach to produce high specificity and affinity antibodies.

As already stated novel antibodies still require animal immunisation. Once the novel antibody is produced its sequence can be determined and any future production can take advantage of non- animal-derived technologies (EURAL ECVAM recommendations, May 2020, EU Directive 2010/63/EU).

82. Immune signalling in infection and cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Immune system, Signalling, Infection, Cancer, Ageing

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To define the role of Hedgehog (Hh) signalling in lymphocytes, Natural Killer (NK) and other lymphoid cells. To characterize the pathway molecularly, and to develop techniques and reagents to modulate signalling pathways to improve lymphocyte/NK function during an immune response against cancer, infection, and inflammation. As part of this, the effect of ageing on the Hh signalling pathway will be examined and the lymphoid response of the aged immune system to tumour and infection.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lymphocytes and Natural Killer (NK) cells are critical to our body's defence against infection and cancer, due to their ability to specifically orchestrate the immune response and effectively kill infected and cancer cells. There are two main types of lymphocytes: B cells, which produce antibodies, and T cells. T cells can be divided into two main classes depending on their function. These are helper T cell (Th or CD4 T cells) which direct the immune response, and cytotoxic T cells (CD8 T cells) which kill targeted cells. The Hedgehog (Hh) signalling pathway has been shown to be important in the development of T cells, the maturation of helper T cells and in cytotoxic T cells for target cell killing. The role of Hh signalling in NK cells and other immune cells is unknown. This work will allow us to gain a greater understanding of the role of Hh signalling and how it functions within the immune system.

Age is one of the greatest risk factors for cancer and the highest cancer incident rates are in older people. Recently there has been much interest in the link between cancer and disease incidence and the ageing of the immune system. By investigating changes that occur to the immune system as individual's age we can gain better understanding of how immune function declines, and more specifically in the role of Hh signalling in these changes.

A comprehensive understanding of the Hh pathway and ageing may suggest new treatment or treatment regimes based on modification of the lymphoid immune response, such as amplifying Hh signalling and potentially increasing the killing potential of cytotoxic T cells in patients with cancer or infections. Such an approach would have the advantage to be applicable to all such patients, since it is independent of the makeup of the tumour or type of infection.

What outputs do you think you will see at the end of this project?

1. A better understanding of the cellular and molecular basis of Hedgehog signalling in immune cells

2. Characterisation of the functional relevance of Hedgehog signalling in immune cells throughout the response to infection, inflammation and tumour challenge.

3. Novel strategies for improving immunotherapy in cancer, infections, and autoimmunity

Who or what will benefit from these outputs, and how?

The outputs from point 1 and 2 will, in the short term, benefit our research group and will help to inform future experiments and build on our knowledge base. In the mid term, the outputs of point 1 and 2 will benefit the wider scientific community through research publications and dissemination of scientific discoveries via conferences. Our research might be of potential use to the pharmaceutical industry. In the long term the outputs of

point 3, and methods for increasing the effector functions of the immune system, may be beneficial for cancer patients. The strategy is not focussed on a specific tumour type or tissue and may also prove beneficial to patients suffering from chronic infection and autoimmunity and may improve vaccination.

How will you look to maximise the outputs of this work?

We aim to publish all our research findings in open-access journals and disseminate our research to the wider scientific community via national and international conferences and lectures. A specific effort will be made to publish novel methods to benefit scientists in the field and also publish data showing a non-significant role of Hedgehog signalling in aspects of the immune system in order to avoid unnecessary duplication of animal experiments.

Species and numbers of animals expected to be used

- Mice: 26500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The immune response is complex and requires a coordinated response from a large number of immune cell types. Many aspects of the immune response cannot be studied in vitro and require in vivo mouse models, for example, the interaction between a tumour and the immune system or the immune response to infection. These involve complex communications between cell types in many organs that cannot be accurately re-constituted in vitro. The mouse immune system is well understood and can be thoroughly investigated since (i) many reagents are available and optimised for its study and (ii) robust mouse models of both malignancy and infectious diseases have been established. The murine and human immune systems are very similar and thus results obtained using mouse models will be directly relevant to human.

We will breed genetically altered animals (GAAs) for use in our studies that will be mainly on a particular mouse background strain (C57BL/6 strain), since most immunological models have been optimised using this strain. The immune system of the mouse does not become fully mature until 6 weeks of age and we intend to study only the mature adult immune response in our experimental mouse models. We therefore do not intent to study embryonic, neonatal or juvenile mice and will only use mice over 6 weeks of age, except in exceptional circumstances. For studies examining the effect of ageing on the immune system we intend to keep healthy aged mice up to approximately 21 months of age. Aged mice will also be on the C57BL/6 mouse strain which has been shown to be long-lived and generally in good health well into old age.

Typically, what will be done to an animal used in your project?

The majority of mice (approximately 60%) used on this licence will be genetically manipulated and used for the purpose of breeding or for tissue collection without any further procedure. Most GAA will be healthy and show no deleterious effects. The mice are maintained in individually ventilated cages in a barrier environment to protect them and ensure any effect of the genetic manipulation on the immune system does not compromise the health of the animal. Approximately 15% of the mice on this licence will undergo regulated procedures that result in the experience of mild distress – such as a single injection or blood sampling procedure. This means that the mice will experience no more than transient discomfort and no lasting harm. The remaining 25% of mice will be used for a short term disease models (usually up to 5 weeks), either a model of infection, inflammation or cancer which may involve multiple procedures - such as multiple injections, blood sampling, imaging and may experience irradiation, intestinal inflammation or tumour burden. These mice may experience a higher level of distress, but in these cases the health of mice will be closely monitored and any mouse showing defined signs of distress and/or poor health will be immediately killed. All animals will be killed by an appropriate humane method at the end of the study.

What are the expected impacts and/or adverse effects for the animals during your project?

GAA for breeding or tissue collection (approximately 60%) and mice undergoing mild procedures (approximately 15%) are not expected to experience any adverse effects, other than transient discomfort, and no lasting harm. The remaining 25% of mice will include animals experiencing irradiation, tumour challenge, infection or inflammation. These animals will be closely monitored and we do not expect them to experience any pain or long-term lasting harm since our disease models are short-term with clearly defined endpoints. Adverse effects will include transient weight loss, and tumour burden which we do not expect to impact on the welfare of the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the majority of our mice (approx 60%) to experience subthreshold severity - used for breeding and maintenance of colonies and tissue harvesting after schedule 1 for phenotyping and in vitro analysis. We estimate 15% of mice may experience mild severity following procedures that produce no more than transient discomfort and no lasting harm. The remaining estimated 25% are expected to experience moderate severity, either a cumulative effect of multiple mild procedures or following a disease model procedure. No animals are expected to experience severe severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune response to antigen, either foreign or self, is complex and requires a coordinated response from a large number of immune cell types and therefore necessitates the need of animal models. The parallels between the human and mouse immune systems are well understood and there are robust mouse models of both tumour biology and infectious disease which can be utilised, with results being directly relevant to human.

Which non-animal alternatives did you consider for use in this project?

To minimise the number of mice used, we intend to carry out as much preliminary work in vitro (culture experiments not requiring living mice) and ex vivo (experiments done in or on tissue removed from the mouse). This will include using established tissue culture cells lines, assessing responses of individual cell types to various stimuli and assessing the signalling pathways in more depth. In addition, we have established a clinical research study using immune cells of human patients and healthy controls. These studies will run in parallel with the mouse studies and will inform and direct the mouse work as well as leading to a reduction in animal use.

Why were they not suitable?

In vitro approaches will allow us to optimise and focus our experiments. However, many aspects of the immune response cannot be studied in vitro and require a mouse model. For example, the interaction between a tumour and the immune system, or the immune response to infection. These involve complex communications between cell types in multiple organs that cannot be accurately re-constituted in vitro and are only sensibly assessed in vivo (within a mouse).

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The majority of the lines in the colony will be maintained on the C57BL/6 mouse strain background which is optimised for immunological studies. This has been used as the basis for our breeding strategies. An assessment of the reproductive performance of the C57BL/6 strain has been detailed in the Laboratory resource: 'Breeding Strategies for Maintaining Colonies of Laboratory Mice' produced by The Jackson Laboratory **(Table 1).**

Table 1: Reproductive information of Jax C57BL/6 strain

Mean Weaning age (weeks)	Optimum Reproductive Lifespan (weeks)	Mean no. of litters born	Mean litter size (born)	Wean:born ratio	Mean litter size (weaned)
4	30	5.4	6.09	0.92	5.60

As standard, our breeding stock will be maintained at minimal levels with 1-2 breeding pairs of different ages at any one time. Breeding pairs are mated typically at 6-8 weeks old and allowed to have 5-6 litters (approx. 30 week breeding period). Replacement breeders will be taken from the stock mice produced or bought in from commercial breeders (for inbred wildtype strains). We try to avoid sibling mating where possible. During the course of the proposed work we estimate we will breed a combination of approximately 18 mouse strains carrying modified immune genes at any time, split between our Mild and Moderate Breeding Protocols **(Protocols 1 and 2).** When colonies are expanded for experimental purposes, breeding trios tend to be used to maximise pup numbers of similar age and provide appropriate littermate controls. However some of our lines give a reduced frequency of GAAs born (approx. 10% compared to 25% expected) and may require additional breeding groups. The majority of mice bred from the expanded colonies will be used for tissue collection for ex vivo work or in subsequent protocols, however there will be the production of unwanted experimental mice which may be used for replacement breeders or culled unused as excess. We currently have 9 mouse lines on each of our mild and moderate breeding protocols - some lines are for breeding purposes only and some lines are expanded for experimental mice. An estimation of the number of breeding females at any one time is given in **Table 2.**

Table 2: Estimated Breeding Females for Mild and Moderate Breeding Protocols

Mild Breeding Protocol 1		Moderate Breeding Protocol 2	
Breeding only (Continuation of line/breeders for experimental crosses) - Breeding Pairs	5	Breeding only (continuation of line/breeders for experimental crosses)	3
Experimental Crosses (Hom x Hom) - Breeding Pairs	4	Experimental Crosses (Hom x Hom)	2
Experimental Crosses (Het x Het) - Breeding Trios	12	Experimental Crosses (Het x Het)	27
Total Breeding Females	21	Total breeding Females	32

From these numbers, we have estimated the number of mice which will be produced from our breeding strategy over the next five years, taking into account that breeding females will be replaced every 30 weeks (a replacement factor of 1.73 a year). This has given us estimated mice numbers for Protocol 1 6500/5 years and Protocol 2 10000/5 years shown in Table 3, allowing an additional 10% for variations in breeding numbers over the five years.

Table 3: Estimated mouse numbers produced on Mild and Moderate Breeding protocols over 5 years.

	Mild Breeding Protocol 1	Moderate Breeding Protocol 2
Active breeding Females	21	32
Replacement Factor (per year)	1.73	1.73
Breeding Females per year	36	55
No. of litters per Female	5.4	5.4
Average litter size	6.09 (born), 5.60 (weaned)	6.09 (born), 5.60 (weaned)
No. pups born per year	1184 (born), 1089 (weaned)	1809 (born), 1663 (weaned)
No. mice born over 5 years	5919	9045
Protocol No (+10%)	6500	10000

For many of our experimental models our initial calculation, in consultation with our Statistician, have suggested we require 7-9 mice per group per experiment. Depending on the controls required for individual experiments, this means the use of an average of 20 mice per experiment, 10 mice per group (control and test) allowing an additional 10-20% per group for experimental losses (such as from tumour engraftment problems or technical dosing issues etc). This will be amended as we gain more information from our experiments and will also vary depending on the number of test groups required per experiment (we may, for example, have a control and 2 test groups in some instances). Most of our proposed disease models are well established and robust, however for some cancer studies, for example, there may be a more variable onset and this may necessitate larger cohorts to demonstrate significant differences. Estimated number of experiments to be performed on each experimental protocol are detailed in Table 4 below.

Table 4: Estimated number of experiments and mice used on experimental protocols.

	experiment	year	year	years
Protocol 4 - Phenotyping	20 mice	4	80	400

Protocol 5 - Immune mice	20	16	320	1600
Protocol 6 - Influenza mice	20	10	200	1000
Protocol 7 - Intestinal mice Inflammations	20	5	100	500
Protocol 8 - Subcutaneous	20 mice	25	500	2500
Protocol 9 - Blood Cancer	20 mice	15	300	1500
Protocol 11 - Cell Labelling	20 mice	1	20	100
Protocol 12 - Skin	20 mice	4	80	400

Protocol 3 involves the production of bone marrow chimeric mice which would be used to address more specific questions and create experimental cohorts of mice from more fragile breeding lines (the chimeric procedure involves the replacement of the immune system with an alternative genotype, by irradiation and subsequent bone marrow transplantation). Mice from this protocol would be directly analysed and we currently estimate creating 10 experimental cohorts on this protocol a year (1000/5 years). Chimeric mice will also be produced to form experimental cohorts as an additional step on other end protocols **(Protocols 5-9 and 12)** within this licence.

We plan to have a continuous staggered ageing mouse colony on Protocol 10. This colony management strategy will allow us to collect samples at varying ages and is also an effective way to manage experiments that require a significant investment in time. It is proposed to initially assign 20 male or female mice in each cohort, based on the number of animals required for an experiment and on the potential loss of animals in a cohort as they age (based on the experience from the current ageing colony which has been running for the past 7 years). We would have ageing colonies for both C57BL/6 wildtype mice and non-harmful genetically altered lines (with matching control mice if necessary) and plan to age approximately 10 cohorts in total a year. We anticipate carrying out the ageing project over the period of the licence, so the total estimated number of mice required on Protocol 10 is 200 mice a year **(1000/5 years).** These healthy aged mice will be used for direct ex vivo analysis. We will also produce healthy aged mice as an additional step on a limited number of other end protocols **(Protocols 5, 6, 8 or 12).**

These are our current estimates for the project licence over the next five years, but will be adapted and refined as we increase our understanding of the field and our breeding and experimental requirements.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing experiments we will initially perform pilot studies to determine the observed effect sizes to ensure that we use the minimum number of mice per group that will be informative. In addition, we will consult with statisticians before starting the experiments and throughout when required to ensure statistical confidence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We intend to run the administration of our mouse strains centrally, using the Laboratory Manager as a central communication point between all users working under the licence and the animal unit staff where the mice are housed. This ensures we can optimise strain breeding to support multiple studies. Should excess mice arise, these can be shared with other researchers who have the authority on their licence to receive them. Collaborating in this way avoids mice being bred and not used, and also avoids duplication of breeding strains within the same facility. The strict control of mouse stock ensures excess breeding is avoided and all mice bred are used as productively as possible.

All mouse lines not in current use are kept on minimal breeding. Where possible, the mice are bred as homozygous lines (providing they do not have a harmful phenotype) and when breeding heterozygotes, littermates are used as controls for experimental work. We have the facility and expertise at the Transgenic Unit to freeze embryos/sperm, and this is will allow us to store those lines needed for future studies but where no research work is planned in the immediate future. On occasion it will be more efficient to study the cell-specific function using bone marrow chimeras. This allows reconstitution of mice with immune cells of a desired genotype and obviates the need to generate new mouse models, to perform complex breeding crosses or try to produce experimental cohorts from lines which produce more fragile genotypes (such as mouse lines where the expression of Hh components have been deleted).

Every opportunity will be taken to decrease the number of animals used for each experiment, whilst still maintaining the statistical significance of the subsequent data. We would undertake pilot studies when using procedures and models new in our hands, to ensure they are optimised before large scale experiments are carried out. To maximise information, multiple body sites will be examined from each animal and multiple analysis types will be conducted on each sample, where possible. Samples can also be archived, e.g. we have optimised cryopreservation of immune cells and created a bank of peripheral immune cells and bone marrow, which can be retrieved and used for future in vitro functional studies, adoptive transfer for in vivo disease models and for production of chimeric mice. RNA and protein samples can also be stored, allowing us to revisit a previous study to perform further analysis without the need to repeat the experiment. This is most important for the healthy ageing colony which represents a large investment in time. We propose to prepare a bank of tissue samples from these mice and collaborate with other researchers interested in ageing to ensure the maximum usage of these scarce mouse populations.

We have setup a clinical research study and have optimised protocols to phenotype and analyse various immune functions in various human immune cells. This will reduce our need for some animal models since work can be accomplished in human immune cells obtained from blood.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen to use mouse models for this project because the murine immune system is well studied and this will allow us to examine our findings in the context of the wealth of information and reagents which are already available and utilise the expertise of other researchers in the field.

Additionally, many murine models of immune cell development and function have been developed and optimised, including tumour biology, intestinal inflammation and influenza. Mice are inbred, relatively small and the mammalian species that is most easily genetically manipulated. They have been shown to yield reproducible results in studies of the immune system. The majority of the GAA we use are designed to be conditional and/or inducible in nature, which will allow us to precisely control phenotype presentation until the candidate gene expression or deletion is induced. This is particularly important since we are interested in the Hedgehog signalling pathway which has important roles during embryonic development.

Why can’t you use animals that are less sentient?

The immune system takes time to establish, develop, and mature in young animals and becomes fully mature only in the mouse from 6 weeks of age. We therefore require the mice to be over 6 weeks of age to study the adult immune system.

Our research is geared towards basic and translational science. In order to be able to translate our findings into the clinic we require an animal species with an immune system very similar to the one of humans. The mouse is an excellent, cost-effective choice in this regard with a wealth of research tools for this species available.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using ex vivo studies allows us to examine the immune system using tissues isolated from mice without the need for further procedures. This will include assays to measure T cell subsets, growth and function, as well as analysis of mRNA and protein expression. The use of in vivo tumour and infection models will initially be refined using these studies. This will allow us, for example, to optimise our experiments and focus on a particular immune cell type or tumour cell line to reduce the number of animals used. Combining the results from the in vitro data and the specific in vivo analysis will allow us to estimate the number of animals required to generate meaningful data, for example analysis of the lung response to respiratory infection will typically require more animals due to the increased variability of the immune response in this tissue compared to lymph nodes.

We have made several refinements to the healthy ageing colony. Originally, only male mice were used, and due to fighting issues, they can end up singly-housed. We have introduced female mice to the colony which can be co-housed at any point to provide company. This will also ensure that any sex- related differences are addressed in our studies. The colony was fed on a high fat diet and C57BL/6 strain mice are susceptible to diet-induced obesity. We are interested in the effect of ageing, and not obesity, on the immune system and have changed the diet of new mice being recruited to the colony to the lower fat diet currently used to maintain our GAA colony as well as the young control mice. This will ensure we are examining only the effect of healthy ageing on the immune system (especially in cancer studies, where obesity has been shown to be a strong risk factor). We will also investigate the option of buying in aged mice from a commercial supplier rather than ageing the mice ourselves. Until now the commercial supply of aged mice has been erratic, but as interest in the effect of ageing increases this has become a viable alternative.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Unless otherwise specified, the work in this project will be undertaken in accordance with the principles set out in the Guidelines for the Welfare and Use of Animals in Cancer Research: British Journal of Cancer (2010) 102:1555-1577 (referred to subsequently as the 'NCRI Guidelines') and in the LASA Guiding Principles for Preparing for and Undertaking Aseptic surgery (2010).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are an active member of our AWERB committee and in constant contact with our NACWOs and animal staff in the animal facility who all have an active role in communicating and advancing the 3Rs throughout the facility.

In addition, we receive regular communications from our Regulatory Compliance Advisor about advances in the 3Rs and relevant conferences and meetings. In addition, we seek advice from the national 3Rs website.

83. Cancer and the microenvironment

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cancer, stem cells, tumour environment, leukaemia, infection

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The projects overarching aim is to better understand cancer development in the context of the tissue which supports its growth and provides chemotherapy resistance.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer causes more than one in four of all deaths in the UK. Despite current therapies many patients diagnosed with cancer die of their disease. Work by our lab and others shows clearly that cancer is highly dependent on non-cancerous cells for their growth, proliferation and chemotherapy resistance. Cancer development is therefore a complex, multi-step process that involves interaction of the tumour cells with the local environment and the immune system and cannot currently be recreated by culture methods. Animal models become necessary because the nature of these pathways and drug treatments require the presence of the cancer and its microenvironment in the setting of a whole animal. These models allow us to study the interaction within the bone marrow microenvironment both in normal physiological processes and in the presence of cancer.

What outputs do you think you will see at the end of this project?

The likely output from this work is to disseminate new information through publication of data in high impact peer reviewed scientific journals. Specifically, the information is expected to subsequently inform the design of clinical trials of new treatment strategies in patients with cancer.

Who or what will benefit from these outputs, and how?

Our research has an established track record in publishing new information in high impact journals.. Moreover, we have also established a track record of bringing our published data to inform new clinical trials. For example, our published work has led to early phased clinical trials and proves biologic rationale for the re-purposing of existing anti-cancer drugs for the use in other types of cancer.

We therefore believe the short term (project year 1-3) benefit from these outputs would be the scientific community as they would benefit from our development and discovery of additional new information regarding the physiology of cancer progression, in support of therapeutic developments.

Medium term (project year 3-5) benefits from these outputs would include the scientific community as described for short term benefits but would also include the pharmaceutical industry who could develop new or existing chemotherapeutic drugs against cancer targets which we identify during this project.

In the long term (post project completion) the ultimate aim would be to improve patient care. We have experience of developing our findings through existing industrial partnerships into clinical trials which may ultimately benefit the patient.

How will you look to maximise the outputs of this work?

Our project team will engage with pre-clinical teams within pharmaceutical and biotechnology industries. Through networking and the presentation of the data at international meetings, we aim to engage with at least two new industrial partners with

drug development expertise. Teleconferencing between partners will be used to communicate our findings.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse is the most appropriate and a widely used model for cancer research. We will use and refine established reagents and protocols to induce cancer. We will look to develop inducible gene expression or deletion of novel candidate genes to study the impact on cancer development with in the context of its environment. Experiments will be conducted on adult mice, including aged mice.

Typically, what will be done to an animal used in your project?

Animals will be given cancer or control cells, with or without non-malignant cells from the tumour microenvironment, through intravenous, subcutaneous or intraosseous injections. These will be usually given only once and the experiment lasts until the animal show symptoms of cancer development.

These experiments will last approximately 30 days.

Before we inject cancer into the animals we need to suppress their immune system. This will be done by using a chemotherapy agent and via intraperitoneal injection. This will be given daily for up to 3 times before injection of cancer.

Once the animals have cancer we will use live in vivo imaging to monitor the cancer development - this will require intraperitoneal injection followed by anaesthesia followed by imaging. This usually takes 5- 10 minutes and the animals are then recovered.

Animals will be mated to establish and maintain colonies for use in cancer studies.

During cancer experiments animals may also be given chemotherapy or other substances by oral gavage, addition to diet or drinking water or intraperitoneal injection. This can be given daily for up to 60 days.

Embryos will be implanted surgically or non-surgically into the reproductive tract to generate to generate offspring for breeding.

Animals will be given microbes or there components to generate an immune response this will usually be given by oral gavage or intraperitoneal injection. The microbes will be given once. The microbe components can be given biweekly for up to 12 weeks.

In some cases blood sampling will be used to withdraw blood for analysis. 10% of blood will be withdrawn and only every 14 days.

What are the expected impacts and/or adverse effects for the animals during your project?

Immunosuppression - moderate weight loss for up to 2 weeks post injection of immunosuppressive agent

Transient pain when performing intraperitoneal, intravenous or subcutaneous injections

Tumour engraftment - Weight loss when tumour is advanced for up to 20% over 72h. If animals show any of the following clinical signs they will be immediately euthanased. These symptoms include rapid weight loss (10% over 24 h), anaemia, laboured respiration, hind leg paralysis, hunched posture or piloerection. Failure to eat or drink over a 24 h period.

Immune activation - transient ill health including piloerection and weight loss. Animals fail to recover over a 24h period will be immediately euthanised

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mating and addition to diet or drinking water will be mild

All animals that develop cancer are expected to have a moderate phenotype It is unlikely that any of these procedures will result in a severe adverse effect

What will happen to animals at the end of this project?

Killed

Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is complex, multi-step processes involving interaction of the tumour and blood cells with the local tissue environment and the immune system, that is set in the context of the immune system, metabolism and physiology of the entire animal. This complex interplay between cancer and the host cannot currently be recreated by culture methods.

Which non-animal alternatives did you consider for use in this project?

We have used in vitro co-culture assays and were possible will continue to use in-vitro experiments as the first proof of principle experiment. Then move the experiments into animals to study the complex interactions of the tumour microenvironment

Why were they not suitable?

These assays are suitable for the first step in identifying interactions that may be important in tumour- host development. However, they don't mimic the complexity of the tumour microenvironment and therefore are of limited use.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have an internationally recognized in-vitro research program from which we have generated as much informative data as we can to limit the necessary in-vivo assays. Together, with our published work describing complex in-vivo experiments have informed us on the number of animals required to answer the objectives of this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The total number of animals to be used in this proposal is primarily based on the use of these established protocols in our previous PPL. These have produced scientifically robust data published in high impact peer reviewed journals. We have combined this with using the NC3R's Experimental Design Assistant which has allowed us to minimise the number of animals required to provide scientifically robust data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will continue to adopt our existing lab approach to minimise animal usage and use appropriate control experiments to answer the experimental question in the most efficient way possible. This will include careful management of colony sizes, the use of surplus

tissue for other purposes where possible. We will optimise cohort size based on scientific evaluation of previous results including in- vitro data. We will perform pilot studies in order to improve the quality and efficiency of larger studies by informing on number of animals required for a well designed study. Other variables we will assess is operator bias and try were possible to use similar age, sex and animals from the same cage. We will minimise the use of mice from single cages.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate and a widely used model for cancer research. For transplantation of (+/- manipulated) cells into host animals we will aim to use the least invasive method the experiment allows to deliver the cells (intravenous before sub-cutaneous/ intraperitoneal before intra-osseous) with a view to minimize the suffering as a consequence effect of the procedure.

For the infection models we propose to study the mammalian response to infection using various bacterial and virial mimics. This reduces the need to use entire infectious agents in the experimental design and allows quantification of the immune stimulant which improves the experimental reproducibility and design, ultimately helping to improve the overall quality of the work and reduce the number of animals needed.

With each batch of infection agent we will assess how dose effects the bone marrow and blood response to these immune system stimulants allowing us to identify quantities necessary to test future hypotheses and ultimately allow us to use doses that minimize suffering.

Why can’t you use animals that are less sentient?

Cancer is an aging disease and thus using animals that are less sentient to study tumour-host interactions would not reproduce the complex biological systems required for simulating these mechanisms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Injection of cancer cells IV - cannula will be small (26 or 27g) and only used once. Monitoring of mice for swelling or hemorrhage.

Blood sampling- cannula will be small (26 or 27g) and flushed with heparin before use to prevent clotting. Monitoring of mice for swelling or hemorrhage.

Choice of agent for mimicking infection - where the outcome is unknown, dosage will be carefully titrated to identify smallest dose that can be used to cause clinical signs of infection.

Animals will be carefully monitored and will be killed by schedule 1 method before they suffer undue effects from the cancer.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

NC3Rs website (https://www.nc3rs.org.uk/experimental-design) will be used for experimental design and the ARRIVE guidelines will be followed to ensure correct reporting of animals research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Review the NC3Rs website (https://www.nc3rs.org.uk)for updates every 3 months. Resources on common procedures will be assessed and advances will be tested in pilot studies to determine their effectiveness in our disease models. Moreover, new advances published regarding experiment design of in-vivo experiments will be analysed in pilot studies to then be applied to setup and experimental disease.

84. Vascular regulation of fibrotic disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Endothelial cells, Liver Disease, Fibrosis, Immune response

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.What's the aim of this project?

Understand the role of vascular inflammation in driving early-stage tissue fibrosis
Determine the contribution of endothelial cells to tissue fibrosis
Assess the regulation of immune cells by the endothelium during fibrosis

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic inflammatory disease is strongly associated with the development of fibrotic disease and ultimately tissue failure in multiple diseases such as hepatitis, arthritis, myocarditis, and atherosclerosis to name just a few. Fibrosis, or tissue scarring, is the most debilitating outcome of chronic disease but currently, transplantation is often the only option for many patients. There are very few if any clinically effective treatments available to combat established tissue fibrosis. The rapid rise in obesity and metabolic disease globally, the most commonly associated conditions leading to chronic liver disease, has led to an unsustainable demand healthy tissue donor for liver transplantation. Currently it is estimated that Non-alcoholic fatty liver disease (NAFLD) affects 13 million people in the

UK alone (~20% of population). Therefore, it is paramount that we develop new strategies to evaluate, diagnose and target tissue fibrosis.
The cells that line all blood vessels, endothelial cells, are one of the first cell-types to sense and respond to tissue injury. Endothelial cells not only sense injury and insult to the body but are also fundamental to wound healing, tissue repair and re-establishing the perfusion of blood by their ability to grow new blood vessel into damaged tissue. Recent studies have revealed that during chronic disease endothelial cells change their behaviour from helping tissue repair to actively contribute to fibrosis. Our work has revealed that a process termed Endothelial-to-Mesenchymal Transition (EndMT) is a key mechanism by which endothelial cells lose their cellular identity and acquire pro-fibrotic characteristics. This work defined new genetic and protein markers for EndMT that were shown to be strongly associated with end-stage liver disease. Single cell RNA sequencing of patients has not only confirmed key EndMT genes are elevated in cirrhotic liver tissue but also highlighted the development of unique disease-associated endothelial populations during the progression of disease.

This licence will focus on understanding how inflammation and metabolic changes, such as high-fat diet, influence changes in endothelial identity. Together the protocols detailed will model different aspects of the complex interplay between the host vascular and immune cell response and provide new approaches to reverse tissue fibrosis and promote the healing and tissue repair.

What outputs do you think you will see at the end of this project?

This project licence will generate new understanding of the role of blood vessels, and in particular endothelial cells, and their wider influence within specific organs and on the body as a whole. The pathways and processes that underlie how endothelial cells change their behaviour following tissuedamage are novel and describing new mediators and signalling pathways involved will of great interest to numerous research fields beyond liver fibrosis. To date endothelial-to-mesenchymal transition (EndMT) has been described to play an important role in a wide range of chronic pathologies including pulmonary hypertension, renal, cardiac fibrosis and tumour progression. The broad appeal of this research will aid in its dissemination through publication in international scientific journals and conferences.

To achieve the objectives of this project we will also generate novel tools and methodologies applicable in the lab, in animal models and potentially in the clinic. To this end we will use the most refined models that can best represent human disease such as inducing chronic inflammation and providing mice with imbalanced diets similar to the high-fat and nutrient-poor aspects of our Western food. By taking these approaches and developing new scientific tools we will enhance our studies application to both basic research and clinical science, forging collaborations and driving the spread of the research.

Furthermore, by understanding the impact on specific tissues, such as the liver, we aim to develop new approaches to identify and treat patients with fibrotic diseases. In parallel with this project licence, we have established collaborative projects that gives us access to clinical samples to validate key pathways identified within these mouse models and develop new ways to assess these processes using human cells in the lab. This will both improve diagnostic and drug screening tests and reduce the burden on animal research.

Who or what will benefit from these outputs, and how?

Steps towards developing new therapies to tackle chronic and fibrotic diseases will bring huge benefits to a huge demographic of patients. However, we clearly have a gap in both knowledge and application before we can attain this long-term goal. This programme of research details a new approach to understand the central and fundamental role that blood vessels play in directing tissue to heal or result in tissue scarring and ultimately fibrosis. Early diagnosis of patients at risk of progressive fibrotic disease is a major challenge facing the clinical management of patients. By characterising the roles endothelial cells during different models of acute and chronic inflammation, imbalanced diet and liver injury, described within this licence, we aim to identify new vascular targets that can be used to diagnosis and potential treat tissue fibrosis. In the time frame of this licence, we intend to understand the key interactions that occur between endothelial cells and the surrounding tissue during the progression of fibrosis with the express hope of mimicking this environment in a dish, such as the Organ-on-a-chip. This will enable us to reduce and ultimately replace animals for screening and validating anti-fibrotic drug targets.

How will you look to maximise the outputs of this work?

This work will be undertaken with a broad range of expert collaborators from both basic and molecular biology to clinical practice. Each step will be validated to ensure that only targets that are applicable to both mouse and human fibrosis are investigated. New knowledge gained from these projects will be presented at national and international conferences while completed studies, irrespective of success, will be published in open-access journals. In accordance with the Concordat on Open Research Data, outputs from publicly funded research will be made freely accessible to maximise use of all data acquired, broaden the research benefits and allow transparent scrutiny of research findings.

We are also keen to disseminate knowledge to lay audiences through public engagement including festivals, imaging competitions and talks to allow public debate about the use of animals in medical research.

Species and numbers of animals expected to be used

- Mice: 3600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This work will be carried out exclusively on mice due to the substantial existing data that describes the extensive similarity in inflammatory and fibrotic mechanisms with humans. The majority of protocols will focus on adult mice to reflect the demographic of patients who develop and present with chronic disease and tissue fibrosis.

To maximise the specificity of studies proposed we will apply inducible genetic manipulation immediately prior to induction of each protocol and enable us to make the most of the wide and valuable array of genetically altered (GA) strains available.

Typically, what will be done to an animal used in your project?

This project will generate genetically altered mice to target and visualise key aspects of blood vessel biology and assess the dynamic changes that occur during models of inflammation, vascular growth, and fibrosis.
In this project we will use some animals in a breeding programme to develop new strains of mice that incorporate fluorescence proteins with specific vascular and fibrotic genes associated with EndMT. These mice will be used in study protocols designed to enhance our understanding of EndMT, provide robust, reproducible, and quantitative measurements of vascular changes, and track cells in real time with both non-invasive and invasive fluorescence imaging. Mice used for breeding will be housed in appropriate living environment that allows them to perform their natural behaviours and maintains their welfare. All genetic modifications will be previously established to minimise any impact on health and wellbeing. Any genetic changes that are known to impact development will be bred with additional 'conditional controls' whereby the gene/s can only be activated or deleted in the presence of additional dietary or drug supplements. Mice assigned to the breeding protocol will not be expected to experience more than transient or mild discomfort.

The four study protocols detailed in this project will assess four key characteristics of fibrotic liver disease, namely inflammation, vascular growth (angiogenesis), nutritional imbalance and fibrosis. To this end, most animals under study protocols will undergo an injection or series of injections of substances inducing inflammatory, angiogenic and liver fibrosis. Study protocols focusing on inflammation and angiogenesis will be conducted for short periods of time with the majority of mice not predicted to suffer more than transient and mild distress. A small proportion of these protocols will also be conducted with short term dietary modifications to understand the influence of these factors on the onset of tissue fibrogenesis. Liver fibrosis models require more prolonged exposure to hepatoxic substances or dietary changes (e.g methionine/choline deficient or high fat) which have the potential to induce clinically assessable adverse outcomes with some mice displaying moderate signs of distress. The vast majority (>90%) of mice in this project will be humanely culled prior to displaying any clinical signs of distress with quantitative data derived following the collection of tissues, blood and bodily fluids.

Approximately 25% of all animals under study protocols will undergo non-invasive imaging to increase the data acquired, refine the monitoring of processes being investigated and reduce the mice required for each procedure. In acute models (up to 2 weeks) animals are likely to undergo the optional steps of having blood taken and undergo 1-2 imaging sessions under 1 hour in length under recovery anaesthesia. For the relatively few mice who undergo procedures longer than 2 weeks; they will be subject to single, weekly anaesthesia to acquire blood samples and undertake imaging sessions of less than 1 hour.

Grouping will be determined by ensuring experiments are designed with comparable control groups that account for genetic background and environmental factors. We will use matched mouse strains to minimise variation between experimental groups and maximise reproducibility. Where applicable we will constrain groups to age, weight, and sex matched animals. This does not restrict groups to single sex animals but makes sure that equal composition of groups is maintained.

Animals that are under procedure will be subject to enhanced supervision to ensure their well-being and ensure that experiments are conducted within the parameters detailed in the licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Almost half of the mice used within this project will be assigned to the breeding programme and will not experience any more than mild and transient distress. By breeding already established genetic altered strains with fluorescent reporter mice and using ‘conditional’ controls of gene deletion/activation we will mitigate any unforeseen impacts that genetic modification could have on normal development and behaviour.

The mice that are assigned to the study protocols will most commonly experience moderate discomfort associated with single or repeat injections of the conditional gene regulators followed by inflammatory, angiogenic or pro-fibrotic substances at doses that are well tolerated. In the unlikely event that mice display localised inflammatory reactions that such as swelling or redness, acute weight loss or abnormal behaviour for more than 24 hours in response to these procedures will be euthanised.

800 mice (22% of the total project) are assigned to receive a hepatoxic substance or high fat diet to induce liver fibrosis. These are more prolonged models lasting >4 weeks that result in clinically assessable liver dysfunction. While these models result in liver damage they are not associated with pain or abnormal behaviour and are only clinically apparent by the release of indicative proteins into the blood steam. Therefore, most mice will only experience mild and transient distress related to substance administration and blood sampling. Mice receiving high fat diet may experience some mild impact on their well-being and condition which will be closely monitor throughout. Mice be assessed by combining defined parameters such as weight loss/gain, abnormal behaviour, clinical signs of liver dysfunction to ensure mice do not experience more than moderate and transient distress. Any mice that exceed our define criteria will be euthanised.

During all study protocol approximately a quarter of these mice will be assessed by non-invasive imaging session/s conducted under short-term, inhaled anaesthesia. Short-term anaesthesia is well tolerated with mice experiencing mild and transient discomfort. To reduce the potential risk of transient respiratory distress, less than 10% of these animals will undergo longer-term anaesthesia (over 1 hour) and these longer sessions will be less frequent if repeat imaging is required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Of the 3600 detailed in this project, 1500 (42%) of animals in this project are assigned to breeding. 2100 mice are assigned to study protocols: 800 (22%) to the study inflammatory models, 500 (14%) angiogenesis models and 800 (22%) for the induction of fibrosis. All animals under used within these protocols will experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

The development of tissue fibrosis is multi-factorial and complex involving both local and systemic interactions between blood vessels, surrounding tissue and circulating immune cells occurring at different times. To fully understand and assess how these different features of biology work together we must apply integrated approaches including assessing cells in the lab, producing computational models and determining how whole tissue responds during the progression of fibrosis. While lab and computational approaches have made great strides in recent years, they are unable to accurately simulate the environmental and cellular changes that occur in living organisms which still provide the most meaningful method of assessing all aspects of tissue fibrosis.

Which non-animal alternatives did you consider for use in this project?

We will be utilising laboratory-based methods such as culturing cells to reduce and replace our use of animal models. Specifically, we will be investigating how organ-on-chip platforms, which can recreate some characteristics of living tissue, can be used to undertake pilot studies of cell-cell interactions, genetic manipulation and pharmacological screening prior to assessing factors in animals.

Why were they not suitable?

One of the biggest challenges is that fibrosis involves numerous processes including different cell types and environmental changes that occur over a prolonged period of time, in patients fibrosis can progress undetected for many years. While we can model key elements of disease in the lab these are only possible for short periods of time. Animal models are the only available method where we can measure the development and impact of fibrosis over a number of weeks to months, making them the most applicable way to model and understand human disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimated number is due to the inclusion of genetically altered animals, which are extremely useful to clarify the role of specific endothelial cells and endothelial-specific molecules in the regulation of tissue fibrosis. These mice need to be bred for several generation before they are suitable for experimentation with 57% of the mice bred projected to undergo a procedure. All possible efforts to reduce, refine, replace the animal use have been and will be made as stated in the 3Rs section below.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This project will use genetically altered, fluorescently labelled, mice to allow us to readily visualise how different cells behave during fibrosis. This approach is a very well-established method that enables us to target selected cells, in this case those of our blood vessels, and image them using non-invasive microscopy. The application of non-invasive imaging will greatly increase both the amount and quality of data obtained from each individual mouse thereby reducing the number of mice required for each protocol. In addition, tissues from the same animal will be used in as many analyses as possible to minimise the number of animals required. Where possible will minimise the number of control mice (those used to compare specific biological response in genetically altered or drug treated mice) by performing internal, comparative controls within the same mice (e.g before and after treatment) thereby reducing the number of mice required per group.

To ensure reproducible and veracity of our experiments we will include randomisation of treatment or control groups, allocation concealment, and blinded assessment to prevent bias analysis of the data obtained. These approaches will reduce the number of experimental groups required and the impact of biological variation between animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The breeding strategy will mainly focus on crossing existing genetically altered (GA) mice with fluorescently labelled, vascular reporter animals. If the existing GA mice are associated with any adverse phenotypes, such as developmental abnormalities, we will utilise inducible gene deletion or over-expression only induce genetic modification in adult animals thereby preventing adverse developmental effect. We will continue to utilise literature searches to confirm we are using the most up- to-date methodologies for the project. This will also ensure there is no duplication with previous reports. Ongoing statistically assessment of quantitative experiments will also enable us to re-assess animal numbers by improving our power analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will exclusively use mouse models of inflammation, angiogenesis and fibrosis in this project. These three study protocols have been chosen as they represent three central roles for blood vessels, and particularly endothelial cells, during the progression of chronic disease. Inflammation is a common process that occurs at the initiation and during active disease, while angiogenesis (the growth of new blood vessels) is a requirement for efficient wound healing and recovery of damaged tissue. The dysregulation of both inflammation and angiogenesis is a characteristic of tissue fibrosis. By assessing them individually, and in combination with clinically relevant metabolic changes such as high-fat

diet, we aim to build a comprehensive picture of how vascular changes can influence liver disease and the progression of tissue fibrosis

Most mice will experience moderate discomfort due to cumulative intervention such as gene depletion and inflammation or fibrosis in order for us to precisely investigate the role of vascular dysfunction. The generation of genetically altered fluorescently labelled mice through our breeding programme will refine our methodologies to allow non-invasive imaging and maximise the data obtained from each mouse.

Why can't you use animals that are less sentient?

This work will be carried out exclusively on mice due to the substantial existing data that describes the extensive similarity in inflammatory and fibrotic mechanisms with humans. There is also a wide and valuable array of genetically modified strains that are being constantly refined to improve the qualityand breadth of data that can used to translate finding to clinical care. Less sentient animals can provide important developmental data endothelial biology however cannot illuminate post-natal vascular response as creatures such as zebrafish and xenopus lack complex immune and fibrotic response restricted to mammals.

The use of mice also provided comparison with this large-scale basic and translational study and extensively studies tools (e.g drugs and antibodies) and pathologic mechanisms that have strong association with developing translational human studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures detail continued monitoring for all animals to ensure that animals requiring post- procedural care, pain management or improved environment stimulation will be addressed. The majority of work will routinely involve brief anaesthesia to minimise the distress associated with the initiation of each protocol. Clear criteria are set to assess animal welfare before and during all procedures for signs of discomfort, weight change and condition will be routinely monitored. The any appearance of these will lead to the animals being considered for early humane endpoints to ensure that no animals suffer unnecessarily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We describe protocols that we have been at the forefront of developing within our research field. We will continue to review our methodologies with other experts within the fields of inflammation, fibrosis and endothelial biology while also seeking the advice and guidance of the animal care staff and veterinary support provided within the establishment. All procedures will follow best practice guidelines laid out by NC3R and LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

All methodologies will be continually reviewed through contemporary literature and by attending national and international conferences to ensure best practice. The applicant and all researchers who work within the protocols described will stay up to date with advances in 3Rs by engaging with expert staff within the establishment and also the wider community via the N3CR website.

85. Environmental adaptation in fish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Natural selection, Evolution, Adaptation, Environmental change, Population genomics

Animal types	Life stages
Fish	adult, gravid, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To quantify adaptive variation and understand its genomic basis in a model fish species.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The natural world is changing rapidly and animals, including vertebrates, will have to move or adapt if they are to avoid extinction. It is critical to humanity that we understand these processes, both for basic, scientific reasons and to manage the consequences of environmental change. The genomic revolution, which has taken place in biology in the last two decades and which allows us to 'read' the 'letters' of an individual organism's genetic code, has massively increased our ability to understand how animals adapt to environmental variation and change. Studies like ours will have significant benefits for our understanding of the way all animals function, including humans.

What outputs do you think you will see at the end of this project?

This project will result in benefits of at least three kinds: publications, information and data.

Evolutionary biology is currently undergoing a revolution in our understanding of the genomic basis of phenotypic variation, as a result of developments in DNA sequencing technology. Simultaneously the Earth's environments are changing rapidly and organisms will have to adapt to these changes. Thus a knowledge of the adaptive potential of organisms is critical to our ability to predict and manage the consequences of environmental change. Hitherto research on the genomic basis of traits in wild organisms has focused on conspicuous morphological differences, but many of the traits required for adaptation to environmental change are physiological, and knowledge of these is in its infancy.

Characterisation of intraspecific variation in physiology is generally poor, and how this is determined genomically is hardly understood. Many important physiological traits have strong linkages to organismal energy usage. There is abundant variation between wild organisms in mitochondrial genetics, but the consequences of this for mitochondrial function are virtually unknown.

We hope to characterise within species variation in critical adaptive phenotypes in a wild vertebrate and to discern the genomic architecture of this variation. Specifically: (i) we will quantify gene expression variation that arises from being reared in salt- versus freshwater, and investigate the genomic loci that contribute to variation in salinity tolerance; (ii) we will quantify intraspecific variation in upper and lower critical temperatures in both wild-caught and lab-raised fish from across our species geographical range; (iii) we will investigate the consequences for e.g. growth and gene expression of rearing fish at different temperatures and assess whether nutritional supplementation can offset these; (iv) we will quantify intraspecific variation in swimming performance;
(v) we will use respirometry to quantify mitochondrial function in fish from different populations and environments. These discoveries will improve our fundamental understanding or organismal function and its genomic basis, especially in vertebrates.

Who or what will benefit from these outputs, and how?

We believe that our outputs will benefit anybody interested in adaptation to environment, and in predicting or managing the consequences of global environmental change, especially evolutionary and fish biologists, aquaculturalists and conservationists.

We will produce outputs of the following kinds:

Publications. We will continue to publish our results in high-quality, international journals, normally at the rate of at least one publication every year. This will begin as soon as results become available and continue throughout the project, likely continuing after the project is complete.

Information. We will disseminate our findings through talks and presentations to the public and scientific audiences on a regular basis: annually at national conferences and biennially at international meetings. This will begin as soon as results become available and continue throughout the project.

Data. All data gathered during this project will be made available on open-access, public databases. Data will be published alongside papers.

How will you look to maximise the outputs of this work?

We will collaborate widely, both within the UK and internationally. We have an established track record of publishing in high impact international journals and presenting our work at national and international conferences. We will continue to do so. We will teach British and international early career researchers, including students, as we have done previously.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our fish, are one of the best models for understanding the genomic basis of adaptation to environmental variation in wild vertebrates. The many novel insights into the genomic basis of adaptation already gained from their study have proven of broad relevance to other species, not least humans. Our fish have a small, well-annotated, high quality genome and well-characterised, genome-wide recombination rates. They are common, easy to catch and easy to rear in aquaria in the lab. They have well-characterised behaviour and occur across a broad array of environmental conditions, e.g. from salt to freshwater and from warm to cold climates. As fish they are the vertebrates with the lowest neurophysiological sensitivity. We are most interested in the phenotypes of fully-developed juvenile and adult fish, hence most work pertains to those life stages.

Typically, what will be done to an animal used in your project?

Typically fish will be exposed to environmental conditions that are within the normal range naturally encountered by the species. Examples include salinities equivalent to freshwater and seawater, elevated and reduced temperatures, high and low calorie diets, high and low current speeds and infection by naturally occurring parasites. In such procedures, exposure to less benign conditions would generally be for short periods (hours to days), but fish might also be reared in more benign conditions for weeks to months. Examples of less benign procedures include exposure to gradually higher or lower temperature, to measure critical temperatures, or to gradually increasing current speed to measure energetic capabilities. Fish may also be housed singly for periods of time from weeks to months.

What are the expected impacts and/or adverse effects for the animals during your project?

The most likely effects on the animals are reduced (or increased) growth rates, increased breathing rates and other chronic, low level stress. These could persist for the duration of experiments. In the case of more serious procedures (critical temperature measurement, energetic capabilities), fish may experience more stress, fatigue and positional disorders.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum expected severity is Moderate, but the proportion of fish experiencing Moderate severity is likely to be less than 25%.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We are explicitly interested in discovering the genomic variation that underlies the phenotypic variation that gives whole animals the ability to adapt to environmental change. Replacement does not apply easily where the animals themselves are the object of study. Nevertheless, within the lifetime of this project licence we may begin to explore cloning and cellular models for the basic characterisation of certain types of genomic variation. These are unlikely to replace the need to understand the whole organism context and consequences of such variation.

Which non-animal alternatives did you consider for use in this project?

In mammalian work, the first steps in characterising the consequences of some types of genetic variation (e.g. coding mutations) can be performed by cloning into appropriate cell lines and investigating relevant cellular phenotypes. We have recently been made aware that this approach might also be possible for preliminary physiological characterisation of genetic mutations in fish. This has been done in some commercial fish species for certain types of mutations, but relies on access to specialised skills and cell lines, and has not yet been done, to our knowledge, in our fish species. Nevertheless, we hope to begin to explore the possibilities within the lifetime of this project.

Why were they not suitable?

The possibility of these techniques requires substantial technological development, and remains speculative. They could not, in any case, replace whole organism phenotypic assays in attaining a complete understanding of environmental adaptation.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we will use has been estimated as the sum of the animals that will be used in the formal experiments that we are likely to do over the course of the licence, plus a small allowance for pilot studies that allow formal experiments to be as efficient as possible. The number of animals used in each experiment has been estimated according to standard principles of experimental design and previous experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We are an established research laboratory, with abundant experience in experimental design and statistical analysis. We are also open to receiving advice from others. We always choose to use methods that reduce the number of animals used, while still obtaining scientifically valid results.

These websites may be utilised to provide additional information on statistics and experimental design: The NC3Rs experimental design assistant https://eda.nc3rs.org.uk/; The 3Rs – Reduction.co.uk site at http://www.3rs-reduction.co.uk/

In order to ensure that high quality, reliable and valid data is produced from the minimum number of experiments, the ARRIVE guidelines (Kilkenny et al., 2010) will be followed when reporting the results obtained from this project.
http://www.nc3rs.org.uk/page.asp?id=1357

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use efficient breeding techniques including, for example, storage of sperm from males when relevant. Small pilot studies will be used when necessary to check experimental protocols and to get an indication of likely results, especially in situations where experimental designs are novel in some way.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Fish are the vertebrates with the lowest neurophysiological sensitivity. The evolutionary response of vertebrates to environmental variations are likely to be qualitatively different from invertebrates because vertebrates possess more sophisticated physiological systems. Humans are also more interested in and attach greater significance to an understanding of vertebrates, partly because of their closer relatedness to us, and partly because we often have a greater interest in the management of their populations.

Fish are the most appropriate species for our work because: (i) they are tractable for fieldwork and common. (ii) They are straightforward to keep and rear in the lab. (iii) Their

population structure facilitates the evolution of local adaptations (iv) They have a fully sequenced and annotated, small genome and outstanding genetic resources. This is highly unusual for a ‘non-model’ organism, but central to much of our research.

In general our project involves exposing fish to either chronic or acute environmental stress (e.g. variation in temperature, salinity, current speed and nutrition). An important refinement is that these stresses will not exceed those normally experienced by the species in its natural habitats. Following exposure animals will be euthanised or returned to optimal conditions facilitating rapid recovery.

Why can’t you use animals that are less sentient?

Our research explicitly investigates the ability of mature animals to deal with environmental variations, and employs assays that require conscious whole organisms, to measure e.g. long-term growth rates or short-term swimming speeds.

It is possible to investigate the fundamentals of adaptation to environmental variation e.g. in insects such as Drosophila that occupy broad environmental niches and have good genomic resources, but extrapolating this to an understanding of adaptation in fish populations of commercial or conservation interest would be difficult.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We use a number of specific refinements in our work in commonly used protocol steps. Marking methods (all protocols): we usually mark individuals using elastomer dye, which is a refinement over fin or spine clipping. It is less stressful to the fish (involving only insertion of a hypodermic needle, and therefore is at the lower threshold), and allows more reliable identification over longer time periods. We always use the finest possible needles (e.g. 30G) for such marking. We are always alert to the development of more sophisticated marking methods. For example, in recent years a number of micro tags (including RFID ‘PIT’ tags) have been developed for small fish, and are now suitable for fish as small as fish. We will consider some of these methods during the lifetime of this project. Any marking methods (including those involving needle insertion) will be practised on dead fish, by anyone without recent experience.

For phenotypic assays (e.g. estimation of critical temperatures and maximum/sustained swimming speeds) we will follow published guidance of best practice. For example, for temperature tolerance estimation we will employ critical thermal methodology, which is a refinement over incipient lethal temperature estimation since the latter involves lethal endpoints and the former does not. In tests of swimming speed, we will test fish in small groups and we will train them first with e.g. slower increments in water speed than used in standard tests.

During normal husbandry, fish will be provided with appropriate environmental enrichment, for example pieces of pipe, where this does not compromise the need to maintain water quality, remembering that many fish naturally occur in water bodies that are often devoid of significant structure although it is also recognised that fish will take advantage of hiding places when alarmed.

Aquarium rooms have artificial lighting with appropriate photoperiod. The temperature of inside aquaria will be maintained between 4 ^{o}C and 20 ^{o}C (depending on season) which substantial experience in other labs has shown to be comfortable for our fish.

Fish caught in the wild will not be housed in tanks with laboratory reared fish, in order to avoid cross infection. Fish will be fed daily on a diet consisting primarily of commercially supplied fresh or frozen invertebrates or appropriately constituted flake. Handling will be minimised, but where necessary fish will be handled mainly using aquarist soft nets. Nets will be chemically sterilised (iodine) between use in different aquaria. Where appropriate fish will be anaesthised after capture, before handling. Abrupt changes of water temperature, pH or oxygen concentration will be avoided.

Water quality is the most important factor in maintaining the well-being of fish and good filtration is the most important tool in maintaining water quality in aquaria. All aquaria have individual, high quality external power filtration systems (except tanks containing fry, where this would be inappropriate).

Power filters ensure good mechanical and biological filtration. Fish are maintained at long-term densities generally not exceeding 30 adult fish per 100 litres. Substantial experience in our lab has shown that these densities allow maintenance of fish health and good growth rates over long periods (months). Ammonia, nitrite and nitrate levels will be appropriately monitored with test kits or meters. Chlorine and chloramine will be removed from water or neutralised before it is used in aquaria. Appropriate amounts of common salt and Sodium thiosulphate will be added to aquaria when they are first established to ensure water quality suitable for fish.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In recent years there has been a surge of new guidance for scientific experiments involving fishes. We will follow general NORECOPA, American Fisheries Society and other guidance (for example the PREPARE guidelines, Smith et al. 2018, guidelines for severity reporting, Hawkins et al 2011, 'Guidelines for the Use of Fishes in Research', Jenkins et al 2014, and ethical considerations, Sloman et al. 2018). We will also continue to follow recommendations in technical publications for best practice in particular procedures. We will develop a monitoring form to facilitate consistent assessment of fish when they are exposed to extreme environments, including signs and behaviours that should be used as endpoints.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By reading relevant papers as they are published, consulting the 3Rs website and attending relevant training courses and workshops (e.g. run by the RSPCA). We will keep in touch with developments in alternatives to in vivo experiments (e.g. Schaeck et al 2013) and with societal considerations about the use of fishes in research (e.g. Message & Greenhough 2019).

86. Dna double-strand break repair, immunity & cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

DNA double stranded break repair, Genome Stability, Immune System, Cancer

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Primary aims of this project are: (1) to develop a mechanistic understanding of the DNA repair machinery that is typically used by mammalian immune systems to generate different classes of antibody; (2) to better understand the pathological role this same machinery plays in cancer development, and (3) to define its function during cellular responses to important classes of anti-cancer drug.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

DNA repair defects in humans can in some cases lead to immune system failure and in other cases an increased susceptibility to develop cancer. This project builds on our previous results that demonstrated that the same DNA repair mechanism that is used to

generate genetic diversity in the cells of our immune system (lymphocytes) is additionally responsible for generating the chromosomal damage and mutations that triggers tumourigenesis in common hereditary breast and ovarian cancer. To date, the cellular mechanisms and DNA repair proteins involved in immune responses and cancer development remain poorly understood, and our research aims to address this unmet need and help scientists and clinicians understand the links between normal immune function and cancer.

What outputs do you think you will see at the end of this project?

The proposed work tackles basic biological questions that are relevant for a number of research fields. This work will define the inner working and principal components of DNA repair pathways that are essential for genetic diversity in immune repertoire, while also describing a molecular process that drives the genomic instability that triggers malignant transformation in the most common forms of hereditary breast cancer. Thus the benefit of this work to the research community will be two-fold: firstly, it will provide new insight into normal immune function, aiding the understanding of human immunodeficiency disorders; secondly, it will reveal the molecular basis of common human cancers, also yielding insight into potential mechanisms of drug resistance that face modern anticancer treatment regimes being used in the clinic. The new insights obtained from this work will also be documented in publications to advance the research field.

Who or what will benefit from these outputs, and how?

This work will benefit patients and the health service. Breast cancer is by far the most common cancer among women in the UK (2010), accounting for 31% of all new cases of cancer in females. It affects over 55,000 people/year in the UK and is responsible for more than 11,500 deaths/year. Commercial beneficiaries such as the pharmaceutical industry may also benefit.

How will you look to maximise the outputs of this work?

We will disseminate our knowledge through scientific publications, and by presenting the data at conferences. We will look to maximize the outputs through collaboration with experts in related and applicable areas. If we find a protein target with therapeutic value we will engage with institute-specific technology transfer department/s and/or the pharmaceutical industry.

Species and numbers of animals expected to be used

- Mice: 18 000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The laboratory mouse is the species of choice for studying immunology, organism level mechanisms of cancer prevention and deriving primary tissues, and the ideal mammal for genetic studies where animals need to be generated rapidly. Using mice provides us with an opportunity to study the role of genes during immune responses, tumour prevention and/or predisposition, and examine their role in genome regulation in well-defined primary tissues, which is not possible in higher organisms. The similarity of human and murine immune systems is reflected in homology at a genetic and protein level, making mice a good model for understanding the equivalent biological processes in humans. For example, one protein we seek to study, REV7 is highly conserved between man and mouse, sharing 98.5% sequence identity.

We make use of adult mice in our experiments due to their developed immune systems. In our development, ageing and cancer predisposition investigations, we will study phenotypes in experimental cohorts of juvenile, adult and aged adult mice, depending on when phenotypes present.

Typically, what will be done to an animal used in your project?

Mice will be bred to produce animals that have genetic mutations that are linked to homologous recombination and non-homologous end joining, or in genes involved in DNA damage signalling responses. These mice will be culled to harvest tissue and obtain primary cells for ex vivo experiments. Some mice will be given immunization challenges which will typically be done by a single injection.

Blood samples will be taken at regular intervals before the mice are humanely killed.

Some of the above mice will possess genetic alterations that are predicted to lead to the development of solid tumours. We will age these mice to monitor for the occurrence of cancers.

What are the expected impacts and/or adverse effects for the animals during your project?

Our breeding programme does not expect to have any impact on the animals.

Our immunization challenges are expected to have minimal adverse effect on the animals due to our use of only well-established foreign antigens and weak adjuvants.

We are breeding and aging some mice that may age prematurely or show predisposition for developing cancer. The main adverse effects will be the occurrence of solid and internal tumours, or a general loss of condition. In both cases, suffering will be limited by humanely killing the animals when appropriate.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

75% mild, 25% moderate.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune response involves multiple, complex systems interacting in a physiological environment, which cannot be replicated in tissue culture, and so there is no adequate alternative but to employ animals in these studies.

In this project, the majority of experiments will be performed using primary cell isolates following purification from the primary tissues of experimental mice. Such experiments will therefore replace the use of live animals in experiments.

Which non-animal alternatives did you consider for use in this project?

Transformed cell lines. Cell culture systems will replace animal tissue in our experiments to characterize the biochemical effects of mutations.

Why were they not suitable?

Transformed cell lines do not accurately recapitulate the properties of primary immune cells in vivo or those harvested from tissues and cultured ex vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We conducted similar experiments on our past project license enabling us to accurately estimate the number of animals we need to use. In conjunction, we have used literature searches, and conversations with collaborators to estimate the number of animals we will use.

We have also used statistics to ensure that we use the minimum number of animals to obtain scientifically meaningful data. Data in the form of various quantitative parameters are assessed by simple statistics or by ANOVA to allow for variable numbers of controls and mice of different age and sex. We are not normally in pursuit of low penetrance phenotypes and therefore do not normally need to examine large numbers of animals.

In breeding experiments, genetic crosses will be carefully designed to obtain the maximum number of useful animals with the minimize wastage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our lab has experience conducting similar experiments on our previous project license which has enabled us to carefully design experiments to reduce the number of animals being used. Experimental procedures are reviewed within our group, and where necessary, consultation on statistics will be sought from our establishment’s genetic statistics service to minimize any factors which could lead to too much spread and a need for more animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure we produce the minimum number of animals to reach our experimental aims, good colony management rules are applied to our colonies using data gathered from a mouse tracking database that is available. We have also previously conducted experiment's with a similar design enabling us to accurately estimate the number of animals we need to answer our research question. Where possible the data from each individual animal will be maximized through the use of tissue for several different experimental aims. E.g. the bone marrow, spleen, and terminal blood samples will typically be collected simultaneously from experimental animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The laboratory mouse is the species of choice for studying immunology, organism level mechanisms of cancer prevention and deriving primary tissues, and the ideal mammal for genetic studies where animals need to be generated rapidly.

Our immunisation method only uses well-established foreign antigens and weak adjuvants, and as a consequence is expected to have mild adverse effects. A single injection and blood sampling is also the most refined and least invasive method for obtaining samples from the mice.

In strains predisposed to developing tumours, a maximum tumour burden limit will ensure that tumours grow sufficiently to obtain scientifically important information, yet not enough to cause distress or suffering. Tailored monitoring regimens in ageing mice will ensure indicators of adverse effects are recognised early to minimise animal suffering.

Why can’t you use animals that are less sentient?

Using mice provides us with an opportunity to study the role of genes during immune responses, tumour prevention and/or predisposition, and examine their role in genome regulation in well-defined primary tissues, which is not possible in higher or less sentient organisms. The similarity of human and murine immune systems is reflected in homology at a genetic and protein level, making mice a good model for understanding the equivalent biological processes in humans. For example, one protein we seek to study, Rev7 is highly conserved between man and mouse, sharing 98.5% sequence identity.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During the immunisation blood sampling regimen, care will be taken to ensure that the animal has stopped bleeding before being placed back in the cage. The mice will be monitored daily for 5 days following immunisation to ensure any adverse reaction is rapidly detected and the mouse killed immediately.

In the case of the aging, development and cancer pre-disposition study, mice will be monitored up to three days a week to ensure early detection of weight loss, loss of condition and/or tumour development. In this way, we will ensure that all humane endpoints are adhered to.

The risk of adverse phenotypes in transgenic mouse strains will also be reduced through use of conditional-knockout alleles in strains harbouring secondary alleles to direct tissue specific Cre- expression. By enabling tissue-specific gene inactivation, such as our proposed use of the Mb1-cre strain to conditionally inactivate DNA repair genes such as Rev7 in early B-cell precursor cells, we will minimize the potential of harmful phenotypes that might occur as a result of gene inactivation in other tissues.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidance published by the NC3Rs, LASA and ARRIVE.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will periodically check whether new guidance's have been published by the NC3Rs or ASPA, and attend any meetings held on the 3R's that take place at our establishment.

Useful websites include: www.nc3rs.org.uk https://science.rspca.org.uk

87. Mechanism of action of vaccines and adjuvants

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Vaccines, Adjuvants, Innate immunity, Adaptive immunity

Animal types	Life stages
Mice	adult, aged, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To improve the design and formulation of new vaccines and vaccine adjuvants through the understanding of their immune mechanisms of action.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vaccines are the most valuable tool against infectious diseases as demonstrated in the most recent pandemic. However, we do not yet have sufficiently effective vaccines against many complex diseases, including malaria. The proposed work will increase our knowledge of how vaccines and adjuvants work. This improved understanding will help inform further development of new formulations to achieve good safety and efficacy.

What outputs do you think you will see at the end of this project?

We anticipate to gain new mechanistic information on vaccines and adjuvants stimulate the immune system. We will publish these findings in peer reviewed scientific journals, and disseminate at scientific conferences.

Who or what will benefit from these outputs, and how?

Vaccine and adjuvant developers in the UK and worldwide will benefit from the knowledge on how different formulations affect the immune response in an animal model of vaccination.

This work uses malaria infection in mice as a model system for evaluating the adjuvant immunogenicity and contribution to improved efficacy. Results obtained will therefore also contribute to the malaria vaccine development efforts within the UK and internationally.

Longer term, this work could lead to the development of new vaccines for complex diseases which would benefit people globally..

How will you look to maximise the outputs of this work?

We have ongoing collaborations within the UK, Europe and the USA through which we exchange and broaden our findings.

In addition to publishing all our results in open source scientific journals, the data will be shared within our scientific networks and presented at scientific meetings and workshops nationally and internationally.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are a well established, cost- and time- effective model for studying vaccines. Mice and humans have a high genetic and physiological similarity, which allows to predict the mechanism of vaccines and adjuvants in humans using this pre-clinical model. The large amount of available data/literature allows more efficient approaches and a reduction in animal numbers. We will primarily use adult mice to establish vaccine immunogenicity, and study mode of action. Some of the work will also explore the longevity of the immune response and vaccine efficacy in aged or juvenile mice.

Typically, what will be done to an animal used in your project?

Novel vaccine formulations will be administered to mice in regimens equivalent to the human routes and dosing. Typically, mice will receive 2-3 immunisations around 2-4 weeks apart. Blood samples will be taken at regular intervals to assess cellular and humoral immune response to the vaccination. In some experiments, vaccinated and unvaccinated mice will be given a malaria challenge to assess the efficacy of the vaccine formulations against disease onset.

What are the expected impacts and/or adverse effects for the animals during your project?

In the majority of the studies the expected adverse effects (e.g. pain) will be mild and transient (maximum 48h post immunisation).

In malaria challenge experiments the severity in some mice will reach moderate. All challenged mice will be screened using blood smears for development of parasitaemia and will be killed before or at the onset of any adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild 60%
- Moderate 40%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Vaccines function at a systemic (body) level. Animal models are the only option to study vaccine immunogenicity and efficacy and mechanistic effects within a whole body system.

Which non-animal alternatives did you consider for use in this project?

There are no non-animal alternatives for systemic studies of vaccine efficacy.

For the studies of vaccine and adjuvant mode of action, we will take a combined approach of using animal models in combination with different in vitro approaches.

We have a parallel project that uses human lymph node tissue which is an approach that can partially replace the use of animals.

Why were they not suitable?

Only answers to very specific mechanistic questions would be possible using experiments on cell lines.

The ex vivo derived human lymph nodes are difficult to obtain and would give limited amount of information on the vaccine and adjuvant mode of action.

The systemic effects of vaccination would remain unaddressed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Each experiment will comprise the minimum number of groups necessary, with each experimental and control group containing the minimum number of animals needed to reach reliable conclusions. We have extensive (over 10 years) past experience with this type of experiments. There are also many published studies which use similar numbers of animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use online tools such as the Experimental Design Assistant developed by the NC3R to help design robust experiments that can provide more reliable and reproducible results. Each experiment will be based on a power calculation to ensure the correct numbers of animals are used. This will ensure the use of a minimum number of animals consistent with the scientific objectives and reduce subjective bias (randomisation, blinding).

We will also use appropriate statistical analysis methods and consult statisticians and bioinformaticians in the experimental design and analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will perform pilot studies to inform and guide subsequent experiments addressing more focused questions.

We routinely maximise the use of tissue (e.g. multiple analyses from the same sample of blood or tissue, harvesting multiple tissues post-mortem).

We also have a parallel project that will employ human ex vivo lymph nodes to study response to adjuvants in human tissue and reduce animal use.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Most of the work will be done on inbred mice which will be vaccinated using common standard clinical immunisation routes. The majority of immunisations will be given intramuscularly as the clinically most relevant route in order to facilitate future translation of this work to the clinic. Intramuscular injection will be done under short anaesthesia, which is the least painful way to apply a vaccine via this clinical route.

For the assessment of vaccine efficacy, we will use a well-established model of pre-erythrocytic malaria challenge. Some mice might experience a moderate severity although animals will be killed before developing the disease. Killing the mice after evidence of malaria infection in the blood (parasitaemia) but before onset of disease reduces suffering and distress to the minimum that is required to assess malaria vaccine efficacy.

Some imaging work will be also be done on anaesthetised animals.

Why can't you use animals that are less sentient?

Adult mice are the least sentient model animals (small mammals) for investigating the general mechanisms of vaccine activity. Some of the work will be investigating the longevity of the immune response and will require maintenance of animals into older age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals will be closely monitored for 10-15 minutes following immunisation and blood sampling procedures. Following anaesthesia, animals will be observed until their return to full alertness and normal behaviour.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines on improving Bioscience Research Reporting.

We continuously review the literature for the newest relevant research which will inform our studies. The local NVS and NACWO are available to advise in case of a new experimental design as well as our Named Information Officer. In addition, we follow updates from the following sites:

www.nc3rs.org.uk https://norecopa.no https://www.lasa.co.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a NC3R’s regional manager available for advice and discussion and attend internal 3Rs meetings.

We also regularly follow the news and updates from NC3Rs and RSPCA and keep our experimental design under regular review for potential improvements in order to increase compliance with the 3Rs. Another useful resource is Norway's National Consensus Platform for the advancement of the 3Rs, http://norecopa.no/

In addition, we continue to look for alternative methods to study vaccines and adjuvants such as human ex vivo models (e.g. secondary lymphoid tissue, fine needle aspirates) and will also consider using cell lines and in vitro systems for more specific/focused questions.

88. Impact of drugs and environment on the brain through the lifespan

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes

Key words

drug addiction, early life stress, genetics, neurodegeneration, development

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this program of work is to understand how genes and the environment interact to affect mental health and brain illness through the lifespan, from early development to old age.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neuropsychiatric, neurodevelopmental and neurodegenerative disorders are widespread, and directly or indirectly impact the lives of nearly all of us. Despite this, most treatments are palliative at best, and in many cases, completely ineffective. It is therefore one of the most critical challenges facing scientists to understand the biology of brain conditions that affect us throughout the lifespan, in order that in the future we are better equipped to treat or even cure them. This project licence will build on work carried out under our previous licence to help to better understand the biology of neuropsychiatric, neurodevelopmental and neurodegenerative disorders.

What outputs do you think you will see at the end of this project?

•This work is expected to provide new information about the biology underlying brain conditions throughout the lifespan, and how this is impacted by genetics and environmental challenge.

•The primary expected benefit is the publication of new scientific knowledge about how behaviours, and particular brain regions and neurotransmitter systems, are affected by the environment in at-risk individuals.

Who or what will benefit from these outputs, and how?

Throughout the life of this project, data produced will be presented at national and international conferences and published in academic journals. The new information will add to the knowledge base about the biology of neuropsychiatric, neurodevelopmental and neurodegenerative disorders. We will also promote and publish any refinements or best practice we identify during this project, thus aiming to improve animal experience and welfare. For example, refinements that we make in our behavioural protocols will be shared, free of charge, to collaborators or interested parties. We have done this in the past, sharing code for our highly refined (non-invasive) working memory test to labs across the world.

In the medium term the pharmaceutical industry will be interested in potential novel therapeutic targets we identify. In addition, in the medium term, we will pass on findings to clinical colleagues, and carry out experiments that aim to translate our findings. For example, as part of a previous project licence, we found that impulsive fish showed greater stress reactivity. That led to an additional grant to my group to work with human participants, examining links between stress and addiction, and recently a grant to work with alcohol-addicted patients and our work on the NIHR research portfolio. We continue to explore this in fish (see Objective 2 and Protocol 2) and will continue to work back and forth with clinical colleagues.

The long-term potential benefits of this study are that data generated may have far-reaching implications for the treatment of neuropsychiatric, neurodevelopmental and neurodegenerative disorders, both in humans, benefitting patients and clinicians by

contributing to the development of effective medications or other therapies, which will ultimately reduce the economic and health burden caused by lifelong neurological conditions.

How will you look to maximise the outputs of this work?

We have several collaborations, both within and outside the UK. These collaborations help us employ a more interdisciplinary approach to our work, and ultimately to publish our work in leading journals. I regularly appear in the media to discuss my research, and present to schools (as part of our outreach programme). This has included demonstrations of zebrafish as a research model to schools, for example. All of our work is published open access. We also release all our data on the open science framework (osf.org) for others to use freely and without limits.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 7700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use zebrafish at all ages, for our research. Zebrafish offer a unique opportunity as a research species, as they are a vertebrate that is genetically similar to mammals, and at the same time offer the offer the potential to keep in relatively species typical conditions (ie in groups, allowing social behaviour). Zebrafish have a fully sequenced, functionally annotated genome, and are extremely robust to mutations. This, in combination with their high fecundity rate (each female can produce ~200 eggs/week), makes them ideal for many questions in neuroscience and biology. Zebrafish become protected (under ASPA) at 5 days-post fertilization (dpf). Many protocols that we use in my group relate to adult behavioural protocols, but we actively work on the development of protocols for younger animals, and even for non-protected (<5dpf) - for example, we have published several studies using 5dpf larvae, and ontogeny studies examining the potential for using younger animals in our protocols.

Our aim is to understand disorders in humans that occur across the lifespan, so we use fish from birth right through to old age (24 months+).

Typically, what will be done to an animal used in your project?

The majority of fish we use are genetically altered, but carry mutations that are not harmful. A typical fish will be exposed to drugs at concentrations relevant to humans during various life stages, to examine the effects on their behaviour (eg learning/memory,

response to novelty, or anxiety caused by a new environment), or on alterations in their neurological responses. Some fish will be grown into old age.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the fish we use will not exhibit adverse effects as a result of any of procedures, or their genetic alterations. Some fish (<1 %) will experience mild behavioural effects such as increased respiration and agitation as a result of the drugs used.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All of the protocols that we carry out are mild. Approx 50% of our animals are exposed to mild protocols, and 50% exclusively to sub-threshold procedures.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The aim of this project is to determine whether life experiences, such as stress or exposure to drugs, alter the coordinated functioning of specific brain areas, resulting in changes in the behaviour of the animal.

The research questions necessitate the use of animals since the influence of stress on the anatomy, physiology and neurochemistry of different parts of the brain and on behaviour can only be elucidated by studying the intact animal itself. To investigate the changes that occur in brain function due to stressors or drugs, it is necessary to expose animals to such conditions and then determine the consequent effects on the brain itself.

Which non-animal alternatives did you consider for use in this project?

We use humans to understand links between genotype, 'risk-taking' personality, acute stress and alcohol use/misuse. This work stems from our work with larval/adult zebrafish carried out on a previous project licence and looks for risk factors for alcohol use and misuse.

Why were they not suitable?

Despite the examples of protocols that can use humans as replacements for adult zebrafish, they are associated with several limitations. Using human participants limits the ability for us to develop detailed understanding of the causal factors relating to the biology of neuropsychiatric, neurodevelopemental and neurodegenerative disorders.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based on pilot studies and our published data. We have extensive experience in using these models and can therefore accurately estimate the number of animals we will need per group to obtain statistically valid results. As we generate more results, we will continually assess group sizes to see whether the number of animals can be further reduced from those currently proposed. We will also use the latest statistical methods for data analysis to reduce sample size.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All our experiments are designed and reported using the ARRIVE guidelines, and with reference to published guidelines (eg Masca et al., 2015; DOI:10.7554/eLife.05519).

1) All our experiments use full randomization and blinding in order to reduce problems with reproducibility in biomedical research. Specifically, animals are allocated at random to all interventions by picking animals at random from several 'batches' (eg tanks of fish nested in clutches of animals) for each experiment. Randomisation is achieved by creating an excel spreadsheet with random numbers, and choosing the tanks in the order they appear. All experimenters and technical staff are blinded to treatment allocation as far as is possible, eg by giving tanks and subsequently individual fish, ID numbers which are noted elsewhere in spreadsheets and not revealed until data analysis stage.

2) We control for possible confounders (e.g., testing tanks) and randomly allocate these as testing units.

3) We account for tank effects in our statistical analysis (ie by adding as a random effect when necessary)

4) Sample sizes are calculated based on a pilot experiment to determine effect sizes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to optimise the numbers of fish that we use for our experiments, we refer to previous experiments to determine effect sizes. For any novel protocols that we are developing, or for drugs that we will use, we carry out pilot studies to determine the expected effect size. In addition, all our behavioural work is fully automated which decreases variability and allows us to be confident in our approaches. Finally, we use environmental enrichment (plants and substrate in the tank) which has been shown to reduce stress, both in our own work and in the work of others. Stress can increase variability in experiments, thus increasing the numbers of animals required (Garner, 2014, ILAR J; Voelkl et al., 2020, Nat Rev Neuro). In order to reduce required numbers of animals, we are very careful to ensure that the animals do not experience stress during their husbandry, using evidence based enrichment and handling methods.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Of all the animal models used in neuropsychiatric research, zebrafish provide a good balance between similarity to humans and limited suffering. For example, females produce many hundreds of eggs per week, which can be extracted in a completely non-invasive manner (unlike, for example, Xenopus).

Zebrafish share genome organisation closely with humans, making them excellent models for genetic research. In addition, and relevant to our work, they share all the major neurotransmitter systems.

Finally, zebrafish show complex and well-characterised behavioural responses.

Why can't you use animals that are less sentient?

It is critical to use vertebrates with a well developed central nervous system (CNS) for neuropsychiatric research. Although we use less sentient animals (zebrafish embryos <5 days post fertilization) for some of our protocols, we cannot use these animals for more complex behavioural testing.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We refine our procedures regularly, including (where possible) replacing invasive with non-invasive protocols. When we carry out any invasive protocols (eg stress-induction, minor surgical procedures) we closely monitor the animals after the procedures, and are eliminating surgery-with-recovery from procedures whenever possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We closely follow the PREPARE guidelines from the NC3Rs (UK), but also the RSPCA and LASA guidelines for animal care.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As well as being members of The International Zebrafish Neuroscience Research Consortium (ZNRC), we receive updates from the NC3Rs (UK) and from our wide network of collaborators across the world. As an example of this, we recently learnt about a less invasive method of genotyping (ID) the genetically altered zebrafish via an NC3Rs publication. We are now working with our technical team to implement the new method. Finally, we have recently purchased a non-invasive larval genotyping machine in order to avoid handling and contacting fish at all during early development.

89. Role of protein degradation in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health orabnormality, or their effects, in man, animals or plants

Key words

Immunity, Ubiquitination, Protein degradation, Infection, Disease

Animal types	Life stages
Mice	adult, embryo, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Proteins are a principle etiological agent of disease. We seek to understand how mechanisms that recycle and degrade proteins prevent or cause disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

By understanding protein recycling and degradation machinery we hope to find ways to inhibit, augment or duplicate these process to treat disease.

What outputs do you think you will see at the end of this project?

The main output of our work will be the dissemination of our novel findings into how protein degradation contributes to disease, its resolution and prevention. This will be in the form of peer-reviewed publications, presentations at scientific meetings and public engagement through the use of press- releases and interaction with mainstream media. Large datasets that we generate will be deposited in publicly available databases, such as PRIDE the PRoteomics IDEntifications database at EMBL-EBI. Any materials or reagents that are generated during the work (for instance, mouse strains, cell lines, antiserum) will be made freely available to researchers upon requested or deposited with central vendors such as the ATCC (American Tissue Culture Collection).

Who or what will benefit from these outputs, and how?

The data we generate during our project will be made available to researchers. Thus those that benefit from these outputs will be anyone with an interest in biomedicine. Specifically, this could include those working, both in terms of research or clinically, on diseases caused by protein deposition and/or misfolding (such as tauopathies) or infections (such as viruses) or metabolic disorders caused by misfunctioning recycling pathways (such as atherosclerosis). This will provide immediate benefit (short term) and continue to impact research and development (long term). A specific longer-term and direct benefit of our outputs we hope will be knowledge that guides the development of new treatments or interventions. We will seek to contribute to this aim directly by testing molecules that have direct effect on disease; either through prevention or treatment. So another way that our outputs may have a benefit is in the development of new therapeutics.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our work by seeking to publish in as high impact journals as we can, to do so frequently and to include as much data as possible. We will also actively seek to distribute our materials and know-how to other research groups, to accelerate others work. We have multiple established collaborations both within the UK and internationally and will use these to both facilitated our work and to disseminate both our successful and unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 47,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will be using mice because they are the model mammalian organisms about which most is known. This is at multiple levels. In terms of biology, we know in most detail in

mice where their biological process are similar, and where the diverge, from us. We have the most experience with mice as a model organism in terms of husbandry and in terms of clinical signs when they are experiencing discomfort so that we can plan suitable and humane end points. Specifically for our research aims, we know that mice conserve the main protein recycling and degradation pathways that exist in humans and so are an excellent model specifically for our area of biology. We have experience working with mice and have been using them as a model for many years in our research.

Typically, what will be done to an animal used in your project?

Typically in our work we will be investigating how mice respond to disease. This disease may be induced by challenging the animals with an infectious agent (eg a virus) or a trigger (eg a prion or prion- like agent) or by inducing a physiological change by activating the expression or loss of a protein in specific tissues or throughout the animal or by delivering compounds or agents that induce or reduce disease. The introduction of these agents may be through injections into tissues or the bloodstream or into the airway. Our experiments are most often short-term, as they involve following the resolution of an acute infection (1-3 weeks). However, other experiments can be longer (1-3 months) in the case of neurodegeneration. The number of procedures are usually limited as we are seeking to understand the effect of a single variable.

What are the expected impacts and/or adverse effects for the animals during your project?

Our experiments involve the study of disease. By definition, this means that normal biological function is compromised. The precise loss of function, and hence adverse effects, are to some degree dependent on the nature of the disease. However, there are common impacts that we use to monitor the animals to ensure their wellbeing and to collect data on the impact of disease. These include weight loss, reduced movement and piloerection. For pathogen-induced disease we expect the duration of these effects to be short (ie < 2 weeks) and to be coincident with the resolution of the disease by the immune system. In some experiments we may use a short-term sub-cutaneous xenograft to report on immune activity but this is not expected to have adverse effects. In the case of more chronic diseases, such as neurodegeneration, the effects may be for longer but in this case we utilise a scoring system to ensure that they do not exceed specific levels.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our research investigates the adverse effects of disease and how this can be treated. By its very nature, most animals in our experiments will experience an adverse impact. The severity of this impact depends on the specific etiological agent. For some viruses, we expect a substantial weight loss that is defined as being a severe impact. In this case we

monitor closely and, using our experience of these experiments, set limits on how long this weight loss should proceed before the animals begin to regain weight. Importantly, it is the likelihood of recovery and duration of adverse effects that we use to determine end-point as well as severity. Overall our experiments cover a range of mild to moderate severities and a wide range of proportion of animals that will reach this end-point (from 1-50%), depending on the disease in question.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The biological processes we study are multi-organ systems, meaning that they cannot be investigated either in mono-cultured cells in vitro or even using organoids. A key aspect of our work is the immune response, which requires movement of cells, antigens and pathogens between different compartments of the body. For instance, we are looking at the T and B cell response to viruses like influenza.

Influenza infects the lung but antigen presentation that leads to activation and proliferation of T cells and antibodies takes place in the spleen and peripheral lymph nodes. Context is important and the manner in which material is trafficked is crucial. Chemotactic gradients are established that are used to recruit immune cells to specific sites of infection whilst antigen enters the lymphatic system and drains through subcapsular macrophages before being presented to B cells. These processes are both multi- component and inter-related and require a level of organisational structure that is currently not possible to achieve outside a living animal.

Which non-animal alternatives did you consider for use in this project?

In vitro cultured cells and organoid models are alternatives we considered. Indeed experiments in these systems are used to complement our work in animals. Where processes can be investigated on a cell autonomous level, we study protein degradation during infection and disease within individual cells.

Why were they not suitable?

We cannot recapitulate the resolution of organ specific disease by combined degradation processes that take place in multiple distributed organs. For instance, how the activation of

T cells in the spleen by antigens that are produced in infected lung cells is impacted by protein degradation and presentation of dendritic cells in spleens and peripheral lymph nodes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals to be used by calculating the smallest possible group size that will give statistically significant data and the number of mice needed for breeding to provide these. The information we require to make such an estimate is two-fold. First, I have held two previous PPLs and have experience in many of the experiments described in this application. We have data on the magnitude of phenotypic responses and the variation between individuals, thus when examining how parameters effect these processes we know how many animals we need to get meaningful results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have a decade of experience with many of the experiments we are performing and where we have less we draw on the published information from others. We use this experience and knowledge when designing our experiments. The key goal is to use the minimum number of animals to obtain a statistically significant answer to our research questions. This is based on accurate measurement of phenotypic magnitude and variation both experimental and biological. One way to reduce numbers is to reduce variability in the data. We do this by ensuring that all those conducting experiments are highly trained in the specific techniques to be used and undergo regularly re-training and assessment. We compare variability between researchers, mouse strains, dose volumes/concentrations, mouse age and sex and all possible variables in our experiments. Where we see a factor is substantially contributing to variation we re-design experiments accordingly. For instance, by changing route of administration of a pathogen.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We adopt a breeding strategy in which we breed animals specifically for experiments. This allows us to reduce the number of animals being bred. We combine experiments where possible so that one control group can serve as the control for multiple parameters being

tested. We take multiple tissues so that we can collect as much data on as many variables as possible within one experiment. For instance, we can monitor B and T cell responses from one experiment rather than setting up two.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our experiments are principally aimed at the study of disease, in most cases as induced by a pathogenic agent such as a virus. The models we use are therefore dictated by the type of infection we are studying. For instance, when we study influenza infection, we use an influenza virus. When we are studying the contribution of a specific aspect of immunity to the resolution of disease, we may use an animal strain in which a specific gene has been genetically deleted. The methods we use in our experiments do not, in of themselves, cause significant pain or suffering as they encompass the introduction of agents through injection or inhalation. The agents themselves, such as viruses, and the immune response they provoke (in the case of immunopathology) are what potentially cause pain and harm. The disease processes we study are those that cause harm and so we cannot avoid this. We do however take steps to minimise this by studying as mild a form of the disease as we can. We also typically undertake short-term experiments so that suffering is minimised. We provide extra husbandry to animals in experiments, such additional food or water or enrichment, to mitigate suffering. We continuously monitor the animals to ensure that the disease is falling within expected parameters and cull animals that exceed these parameters.

Why can't you use animals that are less sentient?

We study mammalian disease and mammalian immune responses, therefore we have to use mammals. Some of the diseases we study only take place in adult animals. For infection studies, we are interested in the responses in individuals with a mature immune system and in weaned animals that no longer benefit from passively transferred maternal antibodies (from in utero or from milk). Age is a parameter in many diseases and infections. Therefore to keep our data internally consistent and in-line with that produced by researchers around the world we use a specific age range of young adults. The exception being in certain neurodegeneration experiments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are always seeking additional measures of disease and the harm it induces and employ both quantitative and qualitative assessments of this. We seek to minimise the number of procedures, eg dosing, required to induce or treat disease. We follow the latest research to determine if new methods, strains or detection systems can be employed to reduce the time of each experiment, the number of mice and the severity of the disease itself (for instance whether a new more sensitive measure can be employed to allow a milder form of the disease to be studied).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We make use of the NC3R website to ensure we use the most refined experiments. We follow current guidance and check the NC3R website to ensure that this is up to date. We refer to ARRIVE (https://arriveguidelines.org) and PREPARE (PREPARE: guidelines for planning animal research and testing. Smith AJ et al) when planning our experiments to ensure we generate meaningful data. Surgery will be performed to best practice as laid out in the LASA guidelines (http://lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf). When substances are administer or blood samples taken, best practice will be followed as set out in A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Diehl et al..

When planning experiments we will refer to Norecopa (https://norecopa.no/) to identify guidelines, search for alternatives and ensure that we are following best practice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We consider the PPL as a living document and our approach is to regularly update it and make amendments. At an institutional level we have a system of regular updates highlighting new findings and training around the 3Rs. We encourage the sharing of best practice between animal technicians and have in place a system of close management and supervision. Our technicians are required to regularly attend formal courses (IAT) and encouraged to pass on new approaches. We analyse the results of each experiment in partnership with both animal and lab technicians. We find this is essential in determining sources of variation and in continuously improving practice and experimental design to reduce the number of mice we use and also minimise the degree and length of adverse conditions. We trial new approaches both in terms of inducing disease but also in following disease outcomes.

90. The integrated stress response in respiratory disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

integrated stress response, pulmonary hypertension

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Cells respond to a variety of stresses by triggering a protective "integrated stress response", which "integrates" different stress signals into a single protective "stress response". We aim to identify components of this "integrated stress response" that when targeted with drugs can treat lung diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

High blood pressure in the blood vessels that supply the lungs (the pulmonary arteries) is a serious condition that affects up to 26 people per million. This "Pulmonary Arterial Hypertension" (PAH) is usually diagnosed in people between the ages of 20 and 60, affecting more women than men. It is a progressive and debilitating disease that without treatment is fatal in half of patients in 2 to 3 years. With treatment, more than half of patients can survive for 7 years, but the quality of their lives can still be impaired and this disease carries a heavy economic burden for both the patient and the health service.

A subtype of PAH called Pulmonary Veno-Occlusive Disease (PVOD) is caused by mutations of a gene called EIF2AK4. PVOD has an even worse prognosis than classical PAH. With no effective treatments apart from lung transplantation, death occurs in 72% of patients within the first year after diagnosis.

Mutations of EIF2AK4 affect a process in cells called the "Integrated Stress Response" (ISR). By studying how defects of the ISR cause PAH in mice, we hope to find new ways to treat this incurable lung disease.

What outputs do you think you will see at the end of this project?

In this project we will investigate the role of the "integrated stress response" in the lungs. We will study how defects in the "integrated stress response" cause some patients to develop abnormally high blood pressure in their lungs, which is also called "pulmonary hypertension". For example, we wish to discover if there is a defect in lung blood vessels in "pulmonary hypertension" or if other cells, for example cells of the immune system, cause lung blood vessels to behave abnormally. This will help us to identify which cells should be targeted by new treatments for "pulmonary hypertension".

All new information will be presented at scientific conferences and published as peer-reviewed research articles. We do not anticipate new products being produced during this project.

Who or what will benefit from these outputs, and how?

In the short term (1-4 years), the main beneficiaries will be other scientists investigating how the "integrated stress response" controls lung blood pressure.

In the medium term, (5-10 years), beneficiaries will include scientists in the pharmaceutical industry who will be provided with new targets for drug development to treat "pulmonary hypertension".

In the long term, we hope to help patients who suffer from "pulmonary hypertension" by giving them access to new treatments that will improve survival and the quality of their lives.

How will you look to maximise the outputs of this work?

All information, including positive and negative results, will be shared with the scientific community at the earliest opportunity at scientific conferences. Once data are of a sufficient quality, peer-reviewed research papers will be published. All research articles will be made freely available via Open Access in accordance with UK funder guidance. All large datasets (including gene expression and protein levels) will be made freely available in publicly accessible online repositories.

Species and numbers of animals expected to be used

- Mice: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This research requires animals that have lungs, an immune system and blood vessels. There is still much we do not understand about how these cell types (lung, blood vessel and immune cells) interact and cause disease. Mice are the lowest creature in which these components can be found. Importantly, mice and humans share many very similar genes, and so we can use mice to understand how defective human genes cause disease. We need to use adult mice because previous studies have shown that many defective genes, including one which we will study called EIF2AK4, cause lung problems that only show up in adult mice.

Typically, what will be done to an animal used in your project?

Mice will be bred to have genetic mutations that cause mild pulmonary hypertension. Although they are unlikely to show obvious signs of disease, we will use non-invasive tests (ultrasound or MRI scans) to determine if pulmonary hypertension is present. In experiments typically up to 6-8 weeks, mice will be given drugs (either in drinking water or by injection) that will either worsen or lessen the degree of pulmonary hypertension. In some experiments, mice will be given drugs to make them unconscious and lung blood pressure will be measured by placing a fine tube into the heart. These animals will be unconscious throughout the procedure and killed immediately after the experiment without regaining consciousness.

Some patients develop pulmonary hypertension because of mutations in certain genes. One of these genes is important in a cellular process called the "integrated stress response". This "integrated stress response" normally protects cells from a wide range of stresses including infections or poisonous chemicals. It now appears likely that a normally functioning "integrated stress response" is necessary to prevent the development of pulmonary hypertension. Therefore, in some animals we will test treatments (by giving drugs in drinking water or by injection) that target the "integrated stress response" to try to

prevent pulmonary hypertension developing or to improve lung blood pressure in mice that have already developed pulmonary hypertension. In some experiments, mice will be exposed to stresses that trigger pulmonary hypertension, such as breathing reduced levels of oxygen (by placing them into a chamber that has lower levels of oxygen than are found in normal room air) or causing inflammation (by injecting them with agents like lipopolysaccharide that trigger the immune system).

These experiments may last up to 18 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts from injection of substances to promote inflammation (such as lipopolysaccharide, also known as LPS) include low-grade weight loss (less than 15% of starting weight). Although we expect some inflammation in the lungs and hearts of treated mice, and that their heart chambers may increase in size slightly, we do not anticipate this to cause the animals distress or breathlessness. We base this on previous experiments in which researchers gave inflammation-causing drugs (such as LPS) to healthy mice and mice with mutations causing mild pulmonary hypertension. No unexpected deaths were seen in either group. Rarely, animals treated with high doses of LPS showed evidence of distress (taking on a hunched posture with bristling of their fur); these were promptly humanely killed and in subsequent experiments we used lower doses of LPS that did not cause these signs of distress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice
- Mild 25%
- Moderate 75%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

Mice are the lowest animal in which an interaction between the "integrated stress response" (ISR), lung blood vessels and the immune system can be studied. While insects

have been used to study the involvement of the ISR in vessel growth, insects do not have lungs and have only rudimentary immune systems that are very unlike those of mammals.

Clinical studies identified the gene EIF2AK4 to be defective in many patients with pulmonary hypertension. At present, how defects of the EIF2AK4 gene cause pulmonary hypertension remains unclear and so effective therapies cannot be developed. Mice and humans both have the EIF2AK4 gene and our ongoing studies have shown that mice lacking this gene develop mild pulmonary hypertension (unpublished work). Using such mice with defective EIF2AK4 genes allows us to examine the role of this gene in lung blood vessel function.

Previous research showed that inflammation can worsen pulmonary hypertension in mice with other gene defects (such as mutations in the BMPR2 gene). We therefore wish to test if defects of the mouse EIF2AK4 gene can also be worsened by lung inflammation. Mice are the lowest animal in which such an interaction can be examined. Although the immune systems of mice and humans share many characteristics, there are some differences. If these differences impair our experiments, we will have access to our institution's Assessment Platform, which provides expert support with "immune- reconstituted ‘humanised’ mouse models" - mice with immune systems engineered to more faithfully model that of humans.

Which non-animal alternatives did you consider for use in this project?

- **Cultured human cells.**

Patient’s smooth muscle cells and endothelial cells from lung blood vessels removed at the time of lung transplantation and donated by the patient for research can be grown in the lab. 'Blood-outgrowth endothelial cells' can be produced from blood samples donated by patients with genetic mutations that cause PAH. We have access to each of these cell models from individuals with normal EIF2AK4 genes and from patients with defective EIF2AK4 genes.

'Reprogrammed' skin or smooth muscle cells from patients can be made to behave as 'stem cells' (so-called iPSCs). These are cells that can be changed into other cell types. Blood vessel cells can therefore be made from these iPSCs. iPSCs can be genetically engineered to have the same EIF2AK4 gene defects found in patients, allowing us to study defects even if patients are unable to donate their cells.

Insects

Fruit flies, an insect model, have been used to examine the effects of ISR defects on blood vessel-like cell development

Why were they not suitable?

- These have been used to study the effects of EIF2AK4 defects on cell function, but such approaches cannot reliably predict the complex interactions between blood vessel cells and other cell types, for example those of the immune system.
- While fruit fly work was useful in pointing researchers towards an interaction between the ISR and "BMP signalling" (a signalling system shared by insects and mammals that controls tissue development), insects lack the complexity of the human BMP signalling system, have only a very rudimentary immune system, and lack lungs.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Previous experiments have shown how a variety of genetic defects and chemical treatments cause pulmonary arterial hypertension. Similarly, previous studies have shown the degree of improvement in pulmonary hypertension that is possible in these animals. With this information we are able to calculate the number of mice needed in each experiment that will provide sufficiently robust results to answer our questions, without excessive numbers of animals being killed.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments will be designed to ensure appropriate numbers of mice are used. Studies will use enough mice to generate conclusive results without being wasteful. To this end, the PREPARE guidelines will be followed to provide a framework for planning studies (see below).

Study design - each experiment will include detail of the groups of mice to be compared (gene defects and / or drug-treatments). For complex studies, a diagram is more easily interpreted. The NC3R's Experimental Design Assistant software will be used to generate plans in which experimental groups can be identified easily.

Sample size - exact numbers of mice allocated to each group will be calculated and stated for each experiment. This will be determined for each experiment depending upon: the reason for the experiment (what is the information being sought?), the size of the difference in the measured readout expected between each group (how big are differences likely to be?), and estimates of how variable any effect will be (how much "noise" will be in the information?).

Inclusion / exclusion criteria - these will typically be defined by animal age and any gene mutations they may have (stated in each experimental plan). Typically, genetic effects will be determined by comparison of mice without gene mutations (so called "wild-type") and genetically modified littermates.

When "homozygotes" (mice with two copies of a mutated gene) are required (e.g. Eif2ak4 mutant mice), heterozygote mice (mice with a single copy of the mutated gene) will be mated with other heterozygotes. When there is clear evidence that heterozygotes (mice with a single copy of the mutated gene) are no different from normal (wild-type) mice, these may be used as controls (carefully recorded) to minimise unused animals per litter. Swift and efficient genotyping (the process of determining whether a mouse has a mutated gene or not) will let us humanely kill mice of a not-needed genetic type at the earliest possible stage to minimise any suffering.

Statistical analysis - careful identification of the "experimental unit" (a mouse) in each experiment will be ensured by labelling to avoid "pseudoreplication" (accidentally remeasuring the same mouse) and "underpowered studies" (insufficient numbers of mice to make firm discoveries). This will avoid inconclusive experiments and avoid the need to excessive repetition.

Experimental animals - surplus mice will be shared between research groups, so fewer mice overall need to be bred or bought. The NC3R's breeding and colony management resources will be used to ensure best practice is followed. To locate genetically modified strains, we will make use of online tools to avoid mouse-strain duplication.

Experimental procedures - close collaboration also lets us share data and learn/use experimental techniques with minimum use of animals for the 'learning curve' – e.g. doses of agents that are known to activate the ISR have been based on preliminary data provided by collaborators in another institution.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Use of non-animal methods and fruit flies (Replacement) will limit the numbers of animals required for the in vivo (live) investigations by helping to pre-select the most effective drugs to test. Experiments will be performed in inbred strains to reduce experimental variability. Where appropriate, we will minimise surplus breeding by avoiding unnecessarily narrow specifications for animal sex, age and weight. We will freeze embryos or sperm when lines are not immediately required for studies. By sharing information with other researchers in this field, we will ensure that different groups are not unnecessarily duplicating similar experiments (except when this is necessary to ensure that research findings are robust). To this end, we will use genetically modified mice (with gene defects relevant to our research) made by other researchers, so that we are not wastefully recreating existing mice strains. In turn, we will ensure that our mice strains are available to other researchers in our field. We aim to use imaging techniques including

echocardiography, CT imaging, and magnetic resonance imaging (MRI) to allow the non-invasive evaluation of heart and blood vessel function. Such ongoing evaluation of the same animal reduces the numbers of animals required in total.

Our previous expertise in using mice to study human disease and the collaborations we have established with experts in related fields will allow the lowest number of animals to be used (Reduction) and sill allow robust statistical analysis. For example, one collaborator previously required 20 animals per experimental group to allow for technical failures, but now obtains sufficient data from groups of 8 animals. Previous experience shows that 10 animals in each control and experimental group provide a 90% chance of detecting a small difference in pulmonary artery pressure or right heart chamber pressure with high statistical confidence.

When there are no appropriate prior results to allow us to design experiments with the necessary statistical strength, for example when using an entirely new drug, we will perform small pilot experiments to provide estimates of the effect size.

For each and every experiment, as part of good laboratory practice, we write an experimental protocol which includes:

• a statement of the objective(s)

• a description of the experiment, covering such matters as the experimental treatments, the size of the experiment (number of groups, number of animals per group), and the experimental material

• an outline of the method of analysis of the results (which may include a sketch of the analysis of variance, an indication of the tabular form in which the results will be shown, and some account of the tests of significance to be made and the treatment differences that are to be estimated)

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The simplest animal that allows us to study the development of pulmonary arterial hypertension (PAH) is the mouse. Genetic mutations that cause PAH in humans have been shown also to cause PAH in mice. Similarly, chemicals that cause human PAH, e.g.

activators of inflammation, worsen PAH in mice. The degree of PAH experienced by the mice we study is typically too mild to cause obvious signs. We can detect evidence of PAH in living mice using ultrasound or MRI scans as would be performed on humans. If necessary, mice can be temporarily anaesthetised (made unconscious with medication) for scans to prevent movement that would prevent accurate measurements being made. For more accurate measurement of PAH, a fine tube is passed through the neck of the unconscious mouse and into the heart. This is only performed when the mouse is fully anaesthetised and the animal is then killed before it can regain consciousness.

Why can't you use animals that are less sentient?

Mice are the least sentient animal in which models of pulmonary function have been developed. The mouse genome has been mapped and so can easily be "genetically engineered" (that is, mouse genes can be altered) to mimic the genetic defects causing human diseases including pulmonary hypertension. Mice also offer the practical benefits of having short breeding times and large litters.

There are many laboratory tools, such as mouse-specific antibodies, that allow analysis of mouse tissues and so enable us to understand the processes occurring in disease and following treatments. Because pulmonary hypertension develops gradually in adult mice, it will not be efficient to perform only "terminal" experiments (experiments in which animals are killed to obtain information). Instead, repeated measurements (e.g. by ultrasound or MRI scans) in the same animals provide more information and so permit the number of animals overall to be kept to a minimum.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all instances, we will choose our models to provide robust data whilst impacting least on animal welfare. Experiments will be designed with an emphasis on animal welfare, being refined to minimise pain, suffering, distress and lasting harm as per NC3R guidance.

Bedding, nesting, enrichment - Mice will be provided with an appropriate environment, e.g. nesting material and shelter, including sufficient space and complexity to satisfy normal murine behaviours. This will be tailored to experiments as required, e.g. translucent environmental enrichment 'yellow- tubes' will be used to enable better observation of the mice while in the hypoxia chamber, removing the need to re-oxygenate them to check for altered behaviour. Additional food and water may be necessary in certain situations. For example, animals show a reduced activity when first placed into a hypoxic environment and so soft food 'mash' and or 'hydrogel' will be added to the cage.

Handling & acclimatisation - Animals will be handled by the researchers routinely performing their care and experimentation in order to minimise distress from unfamiliar experiences. When repeated or prolonged interventions are required, animals will be

acclimatised to new environments (e.g. hypoxic chambers) or procedures (see non-invasive approaches below).

Observation and monitoring - Animals will be monitored at least daily and more often following interventions as appropriate. For example, injections will be carried out in the first half of the day and animals will be monitored for signs of adverse effects for a period of 1 hour after drug administration and will be monitored regularly for signs of adverse effects within 24 hours of administration, e.g. bristling of the fur, hunched posture, subdued behaviour, reduced response to stimulation, or diarrhoea. Any animal will be immediately killed humanely if it shows evidence of suffering that is greater than that specified in the relevant protocol. When animals will be placed into a hypoxic environment, they will be monitored closely at least twice-daily for adverse reaction to the intervention. Animals will be weighed three times per week during the hypoxic period and weights recorded. The respiration rates of the animals will be monitored closely and changes noted. Pilot studies will be used for unfamiliar or novel procedures to establish experimental and humane endpoints, and we will perform post-mortem examinations as a routine part of all pilot studies to investigate any unexpected deaths, seeking advice from the named veterinary surgeon as needed.

Amelioration of pain - When procedures are performed, e.g. vasectomy, painkilling medication will be provided during and after the operation, as agreed in advance with the named veterinary surgeon.

Procedures will be performed using anaesthesia whenever appropriate. This will be regularly reviewed by a veterinarian to ensure that contemporary best practice is followed. Animals will be allowed to recover before further use, e.g. following vasectomy mice will not be used for mating until they are regaining weight and they are showing no adverse signs following surgery.

Administration of substances and removal of blood - We will follow the guidance of the European Federation of Pharmaceutical Industries Association and the European Centre for the Validation of

Alternative Methods. For injections and sampling, needles / catheters will be of the minimum width effective for each purpose. Adverse effects from administered substances will be limited by administered using the minimum effective volumes and frequencies to minimised discomfort, and using the lowest effective doses. Drugs will be dissolved in solutions that have been tested and deemed safe. Repeated administration may produce repeated mild distress. If the cumulative effects threaten to exceed that specified in the relevant protocol, the advice of the named veterinary surgeon will be sought and if necessary, the animals will be killed humanely. For blood sampling, no more than 10% of total blood volume in 24 hours and 15% of total blood volume in any 28 day period. Animals will be weighed to ensure bleed volumes are not exceeded. Lipopolysaccharide (LPS) is a chemical that triggers the immune system and will be used in some experiments to increase pulmonary hypertension. It will be administered following existing Home Office

guidance, in particular taking into account age, sex and strain differences in LPS effects (older animals, females and outbred strains being more sensitive) with dosing being adjusted accordingly.

Non-invasive approaches - We will use "non-invasive approaches" (methods that do not involve placing tubes into the mice) where-ever possible. Lung blood pressure measurement using ultrasound scanning is possible for mice just as it is performed for human patients. Ultrasound provides measurements that similar to lung blood pressures measured directly by inserting tubes into the blood vessel, but has the advantage that it allows repeated monitoring of the same animal over several weeks, thereby reducing variability in the experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As detailed in the **Guidelines for the Welfare and Use of Animals in Cancer Research Apply (2010),** we will use all available knowledge to predict adverse effects and provide specialist care, especially for genetically modified animals.

Laboratory Animal Science Association (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) will be used to guide aseptic technique for surgical procedures (both recovery surgery and non-recovery procedures carried out under terminal anaesthesia) to ensures surgical procedure are carried out skilfully with the minimum of risk and disturbance to the mice and without infection.

Animal Research: Reporting In Vivo Experiments (ARRIVE 2.0, 2020) guidelines checklist will be used to ensure optimal reporting of data from mouse experiments can be fully evaluated and utilised. The guidelines are aimed primarily at scientists writing up their research for publication and for those who are involved in peer review

Experimental planning will follow the guidance set out in the **Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (PREPARE)** document (Smith et al 2018 Laboratory Animals, 52(2): 135-141).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs both by regular updated provided by our institute and from the NC3R's webpages e.g. resources page (https://www.nc3rs.org.uk/3rs-resources). We will make ongoing use of the NC3R's E-learning hub (https://nc3rs.org.uk/e-learning-resources). Refinement resources will also be accessed. Resources from the Laboratory Animal Science Association (LASA) will also be reviewed as updated, e.g. the guidance on dose selection in toxicology studies https://www.lasa.co.uk/PDF/LASA-NC3RsDoseLevelSelection.pdf will be useful to our programme of work.

91. Phenotypic plasticity and adaptation of cichlid fish in response to novel environments

Project duration

3 years 0 months

Project purpose

- Basic research

Key words

Adaptive evolution, Phenotypic plasticity, Epigenetics, East African cichlid fish, Environmental perturbation

Animal types	Life stages
Haplochromine cichlid fish of the East African Great Lakes	adult, neonate, embryo, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To characterise the impact of dietary change in East African cichlids.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In order to protect global biodiversity it is essential to characterise the impact of environmental changes on a range of animals. Research in the past few decades has established that changes in diet and temperature can lead to changes in parts of the body without requiring changes in DNA. These changes are referred to as phenotypic plasticity

and can result in rapid adaptation of parts of the body and behaviour. Understanding the molecular basis for these changes is crucial to predict how animals will respond to a shifting climate.

Fluctuations in environmental conditions often lead to changes in available food sources. This can result from a range of factors including altered prey populations, vegetation dispersal, and migration patterns. In aquatic systems, changes in the quality of the water are leading to the rapid decline of microorganisms crucial in maintaining the marine food chains.

For instance, measuring the abundance of phytoplankton - a type of small aquatic plant - can indicate harmful effects of environmental change in aquatic environments. The upper water column, or shallower level, of the ocean has warmed ~0.1C per decade throughout the past 40 years. Linked to this warming, studies have measured a global decline in phytoplankton levels over the past several decades, as well as changes in their dispersal and yearly life cycles. Because phytoplankton make up much of the basis of the aquatic food chain, changes in the abundance and locations of these small organisms can impact every level of the aquatic environment, placing resource strain upon a range of animals. Currently, the effects of such a widespread change remain uncertain.

For this reason, we are simulating altered dietary conditions in a fish model to study the short-term adaptive process. In the proposed research, we will use East African cichlid fish—a fascinating system known for their rapid adaptation to novel environments—to investigate the responses to changing environments. Our research has two main aims. First, we will characterise the changes that occur in response to altered environmental inputs (e.g. diet) across multiple generations. Second, we will determine whether these changes persist in future generations after returning to the ancestral environment.

To address these two aims, a population of juvenile Astatotilapia calliptera bred from stock tanks will be randomly divided among three diet treatments: a marine diet, an algae diet, and an omnivorous commercial food that is normally fed to this species (referred to as the ‘control’ diet). Fish from generation 1 (G1) will be bred within their tanks to produce generation 2 (G2). The G2 offspring under treatment will be randomly divided and will continue on either the marine or the algae diets. Throughout subsequent generations, all fish will return to the omnivorous ‘control’ diet. Relating to objectives 1 and 2, objective 1 is fulfilled by G1 and G2, objective 2 is fulfilled by all subsequent generations.

What outputs do you think you will see at the end of this project?

The outcomes of the proposed work have the potential to result in several publications reviewed by other scientists. These publications will report findings related to the following main topics:

1) Identification of genes involved in diet adaptation across several parts of the body, including muscle and liver tissue.

2) Characterising how diet change alters body parts in the absence of genetic change and determining the molecular processes behind those changes.

3) Determination of whether non-genetic changes as a result of altered environment are inherited after returning to the original environment.

We expect the data generated in our work to be useful to other researchers and lead to collaborative work. We will deposit our data in publicly available, free repositories, so that other researchers can download it and use it to answer their own biological questions. This is also a reduction measure, as it should avoid repetition of the same work by other researchers.

Who or what will benefit from these outputs, and how?

Our work is of a fundamental nature and will build important knowledge of the impact of altered environments on the control and usage of genes. This will increase our understanding of short-term adaptation in the absence of changes in DNA. As our project touches upon so many fundamental biological aspects, we expect our results to impact several fields of the biological sciences, e.g. evolution, molecular biology, and genetics.

Given the fundamental nature of the proposed work, the research output is not likely to produce directly any product or method with commercial potential. However, the impact of altered diet on fish growth and reproductive success may be used to enhance productivity in commercial fish farming by informing the composition of fish food designed for optimal growth. For instance, as this project involves feeding a population of fish multiple different diets, one of these diets may result in a significant increase in health and growth rate. This finding would suggest that a similar nutrient composition may lead to increased commercial output in fish farming.

How will you look to maximise the outputs of this work?

We intend to present our results in local and international scientific conferences or other scientific meetings. We will attend at least one major scientific conference per year on topics related to our proposed research. These conferences will provide opportunities to broadly communicate our results. Collaborators and other persons directly involved in this work will also participate in dissemination efforts.

Our investigations have already led to several new collaborations with other researchers. We will disseminate all findings, including negative results. Our work will be published in free, public repositories online and in international scientific journals.

Species and numbers of animals expected to be used

- Fish: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using East African cichlids of the African Great Lakes due to their unique and fascinating diversity of body sizes, shapes, diets, and behaviours. These characteristics influence each other and together indicate how well prepared the fish is to deal with and explore its surrounding environment. In fact, the diet of these fishes strongly influences the shape of their skulls and jaws. The combination of these features make East African cichlid fishes, as a whole, an ideal system to identify the genes underlying the bodily changes relating to diet.

In order to understand the effects of a specific diet, these fishes will be fed the same diet throughout their entire life, from the time they initiate independent feeding.

Typically, what will be done to an animal used in your project?

The environmental variable perturbed in this experiment is diet. The diets of the animals in this project will be altered to represent the food regimes of different fish in the lake. Specially formulated fish feeds have been designed to represent the diet of algae eaters (algae-eating diet) and of fish eaters (fish- eating diet) while preventing any malnutrition in our experimental group.

Two groups of fish will be fed two distinct carefully determined diets. One group of fish will eat an algae diet, while another group of fish will eat a marine (fish-eating) diet, both in the form of pellets.

A control group of fish will eat the conventional diet of our aquaria stocks, in the form of flakes.

What are the expected impacts and/or adverse effects for the animals during your project?

We carefully defined each diet so that all the nutritional requirements are met. Thus, we expect our proposed procedures will impact the animals' health and welfare minimally and will not lead to long- lasting pain, suffering, or distress.

In the cases where the diet change produces effects at the level of the skull and jaw, the animals will be killed if their feeding, reproduction, or behaviour are impaired to a point inconsistent with a healthy and normal life. Animals whose diet strongly affects their feeding, reproduction, or behaviour, will be humanely killed immediately to prevent any pain, suffering or distress.

The conditions of the animals will be monitored daily. Weight and length will be recorded once every two weeks to ensure the animals remain at healthy levels. Tanks will be cleaned at least once a week and water parameters will be measured regularly. In the

case that tanks need to be cleaned more often to maintain optimal water parameters, for example when fed with one of the altered diets, we will incorporate this change for all tanks involved in this experiment. No adverse effects are seen from additional cleaning.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Fish: Mild severity: (less than 10%).

What will happen to animals at the end of this project?

Killed
Kept alive
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is important to understand how animals react to a changing environment, and in particular to dietary changes. To research this topic, we need to feed animals with particular controlled diets.

Which non-animal alternatives did you consider for use in this project?

We have considered computer simulations and cultures of cells.

Why were they not suitable?

At the moment there are no alternative resources capable of recreating the natural development of fish. In order to fully understand how animals react to a changing environment, we need to observe how animals develop and behave in a natural context. Other alternative systems, such as cell culture, or computer simulations, are not able to recreate the complex interactions that occur at the level of the organism and during development, between neighbouring cells and tissues. These interactions are most important for proper animal development. Thus, our aim cannot be accomplished without using animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We plan to produce four generations of fish throughout this experiment (maximum of 1500 animals):

1. In order to produce juveniles for the first generation (G1) of the experiment, we will breed our stock fish of that species. We expect to use a maximum of 30 stock animals to produce a founding population of 360 fish. We will divide the founding population across three treatments.

2. For the second generation (G2), we aim to produce 370 fish through controlled breeding of G1. We will include at least 120 animals per treatment.

3. For the third generation (G3), we aim to produce 370 fish through controlled breeding of G2. We will include at least 120 animals per treatment.

4. For the fourth generation (G4), we aim to produce 370 fish through controlled breeding of G3. We will include at least 120 animals per treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A pilot experiment was performed using a smaller subset of fish and commercial diets of similar types to the ones created for this experiment. The results from this pilot verified that significant gene regulatory changes can be observed within one generation of diet perturbation in this fish.

Meetings with collaborators who performed similar experiments in other fish models assisted us in determining the minimum number of fish required for significant sampling power. The diets will be meticulously defined in order to meet all the nutritional requirements of the fish. These measures ensure that the experiment will not have to be repeated in a large scale.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Throughout the duration of the project, we will use the minimum number of animals required to acquire satisfactory data. Experiments will be designed, conducted, and reported according to published guidelines (namely the PREPARE and ARRIVE guidelines).

The pilot experiment, mentioned above, allowed us to define an adequate experimental design in terms of diet and number of animals used. The fishes from the founding

population will be randomly assigned to experimental and control groups. A range of tissues will be dissected from killed animals, and those tissues not immediately used for the purpose of this project will be available to colleagues and collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use East African cichlid fish for the proposed work plan. As these fish species are large and robust, they can be effectively bred and grown in an aquaria environment. Moreover, these animals can tolerate novel diets with no major biological consequences. Thus, these dietary experiments are expected to cause little to no pain, suffering, and distress to the animals.

Why can't you use animals that are less sentient?

The work proposed here takes advantage of the extreme diversity of shapes, sizes, colours, diets, and behaviours of East African cichlids. The diversity of these cichlid fishes is unique amongst vertebrates and even amongst the entire animal kingdom. Therefore, other less sentient, or invertebrate, animals will not allow us to answer the same biological questions.

Tissue dissection and extraction will be exclusively performed in dead animals.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will incorporate new recommendations on animal welfare, as soon as possible after their publication.

Fish will be checked daily for any adverse effects of the diet treatment. We will alter the level of food given to the fish on a regular basis to ensure the fish are consuming optimal levels for their life stage. Additionally, the algae and marine diets, both in pelleted form, will be ground up to smaller sizes for younger fish to ensure they are as easy to consume as possible. It is important to note that food pellets soften upon contact with the water so, at any life stage, remain easy for the fish to consume.

Lengths and weights of the fish will be measured every two weeks to ensure they remain at healthy levels. These measurements will be taken with minimal stress to the fish as they remain in water throughout the procedure. No anaesthesia will be used when weighing or measuring. The weights and lengths of fish under the algae and marine diets will be charted over time to determine growth rate.

These rates will be compared to those of fish consuming the omnivorous parental diet (referred to as the 'control' diet) and comparisons will be used for monitoring purposes, including surveys of overall condition via body mass and comparing growth rate to that expected for this species (i.e. growth rate of the omnivorous population). Body mass of the fish will be assessed relative to breeding frequency to determine optimal endpoints for each generation.

Cichlid housing will be continuously improved to faithfully mimic the sandy areas rich in vegetation and hiding places characteristic of their natural environment. This will be achieved by introducing sand, artificial plants, clay pots, and plastic tunnels in the aquaria. Fish will be housed in groups, in order to decrease stress and aggression levels. Manifestations of aggression, including chasing, fighting, and injuries will be monitored

daily by the scientific and animal care staff. These aggressive behaviours are normal to these species and not a product of captivity. If required, fish will be temporarily separated and groups redesigned to avoid further injury and aggression. Continuous monitoring of water parameters, food regimen, and breeding will ensure the wellbeing of the animals.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the best practice guidelines available at www.nc3rs.org.uk. Experiments will be planned, documented, and reported according to published guidelines (namely the PREPARE and ARRIVE guidelines). In accordance with suggested practice for fish models, enrichment will be added to each tank and individuals will be housed in social groups to minimise stress and increase quality of life.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will follow the website of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs, available at www.nc3rs.org.uk), and stay up-to-date with new information and new resources that become available. To achieve this, I have signed-up to the NC3Rs e- newsletter.In addition, named persons at my establishment (i.e. the Named Animal Care and Welfare Officer, Named Veterinary Surgeon, and Named Information Officer) will inform us of relevant new information and resources, and will advise, together with the animal care staff, on the best ways of implementation.

92. The impacts of marine engineering on wild fish

Project duration

5 years 0 months

Project purpose

- Basic research
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Marine fisheries, Telemetry, Marine engineering, Windfarms

Animal types	Life stages
Atlantic cod (Gadus morhua)	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to investigate how marine offshore windfarms affect wild fish movement and habitat use and whether they have potential to enhance economically and culturally important commercial and sport fisheries.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Offshore windfarm (OWF) development is the fastest growing energy technology in Europe and is anticipated to double by 2025 with thousands of turbines constructed around the UK coastline. The foundations and scour defence structures of these turbines form artificial reefs which alter marine ecosystems and biodiversity. It is thought that these sites provide productive feeding and breeding sites for numerous fish species, some with commercial

and cultural value as food fish for human consumption and recreational angling. Therefore, these sites have potential to increase stocks of fish species and in turn benefit fisheries and economy, an effect known as ‘spill-over’. We aim to record the movement of five commercially and culturally significant fish species within and around OWFs so that we can assess spill-over and the management of fish stocks that interact with OWFs. Information on the ecology of fish, including their distribution and habitat use under a changing environment will help inform government departments and agencies and engineering companies in planning and designing marine engineering schemes, such as OWFs.

What outputs do you think you will see at the end of this project?

The project will generate fish tracking data by using acoustic telemetry devices which emit unique sounds (pings) that are picked up by receivers placed around offshore windfarms. These tracking data will be combined with sea-bed survey, GIS data and statistical modelling techniques to estimate the amount of time fish spend within the OWF and define specific areas (habitats) of high fish activity within the OWF. The movement and habitat use data will be analysed and written as original research papers for the scientific community in open-access peer reviewed journals and scientific conferences. Our analyses will also be presented to and shared openly with OWFs and government bodies to inform planning and policy with regards to OWF development. They will be shared through a workshop with key stakeholders and a short video published through the European Regional Development Fund "ECOSTRUCTURE" project webpages and social media platforms.

We will be using wild marine fish in our research. For some of these fish species there is limited knowledge on anaesthetic and analgesic process when performing regulated procedures, such as telemetry device implantation. Therefore, added value of our project is the reporting of optimal care and handling of wild fish to minimise pain distress and suffering. We intend to report these aspects of the project via standard peer-reviewed publication and the NC3Rs and Norecopa portals.

Who or what will benefit from these outputs, and how?

The building of offshore structures for harvesting energy is happening at an unprecedented rate but we don't yet fully understand the impacts of these structures on fish. Over the duration of the project (mid-to long-term) OWF developers and planning groups will benefit from our data highlighting the impacts of OWFs on fish movement and use of the artificial habitats that these developments create. This information will assist developers and planners in considering how to shape habitats when designing OWF foundations and scour defences. These impacts will extend to the longer term as data collection on the project is fully realised and relayed to government authorities, conservation bodies and the general public. Stakeholders outside of the OWFs include commercial & recreational fishing operations and national and international regulatory bodies concerned with assessing and protecting fish stocks and marine ecosystems. The

information will also contribute to the development of methods for assessing management of fisheries and the impacts of OWFs.

The scientific community will benefit from the refinements we make in the procedures on wild fish for surgical implantation of tracking devices and in-field handling of these species.

How will you look to maximise the outputs of this work?

Results of the project will be shared with stakeholders with the intention of providing an understanding of how OWFs may be important for fisheries, and how current legislative and conservation efforts may be adapted to include these effects. Furthermore, a short explanatory video will be produced and published across the European Regional Development Fund 'ECOSTRUCTURE' project social media accounts, scientific conferences and meetings with stakeholders. The video will focus on the context on the project, the technology used in the project, and the results. The outcomes of this project will also be published within open access journals and at scientific conferences and elements of it shared with the NC3Rs and Norecopa.

Species and numbers of animals expected to be used

- Other fish: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The fish used will all be adult wild-caught marine species. There is no alternative to using live fish because we aim to study the influence of OWFs on wild fish ecology and behaviour (i.e., movement and habitat use). We will study a range of species, including for example, cod (Gadus morhua), thornback ray (Raja clavata), bass (Dicentrarchus labrax), pollock (Pollachius pollachius) and ballan wrasse (Labrus bergylta). We will use adult fish to represent the breeding stock and to minimise harm by using individuals of a size easily capable of tolerating small tracking (acoustic telemetry) devices.

Typically, what will be done to an animal used in your project?

Fish will be caught directly from the sea using rod and line fishing techniques on a commercial angling boat and held in large on-board seawater tanks to assess their health against established condition criteria. At this point fish will be assessed for their health and suitability for inclusion in the study. We anticipate very few fish (<10%) will not meet the condition criteria for inclusion in the study because we will use angling techniques that minimise trauma and distress to the fish. Any fish caught, but that do not meet the necessary condition criteria for surgery will be recorded but not included in the study. For those fish deemed fit and suitable, we will insert small acoustic telemetry devices into the

body cavity through a small incision and under general anaesthesia and with local pain relief. For thornback rays, telemetry devices will be attached externally using special tags inserted into the 'wing' (pectoral fin) of the fish under sedation and local pain relief. At the end of each surgical procedure, fish will be assessed by appropriately trained and qualified persons to ensure that the fish are fit and their welfare protected. Tagged fish will be released into the wild for monitoring and therefore not deliberately recovered/recaptured. Each fish will be operated on only once. We anticipate tagging 50 fish of each species being studied (up to a maximum of 250 fish in total) and will record movements of the released fish for up to three years.

What are the expected impacts and/or adverse effects for the animals during your project?

Any surgical procedure on an animal has potential to cause pain and physiological disturbance as well as distress. We will minimise these possibilities as follows. Only fish deemed fit and suitable for inclusion in the study will be operated on. All fish will be handled, assessed and operated on by highly skilled individuals capable of quickly establishing fish health and recognising signs of distress.

Insertion of telemetry devices into the body cavity (peritoneal cavity) will be done through a small incision and under general anaesthesia and with local pain relief. For thornback rays, telemetry devices will be attached externally under sedation with an anaesthetic and with analgesia to minimise operative and post-operative pain. At the end of each surgical procedure, fish will be assessed by appropriately trained and qualified persons to ensure that the fish are fit and their welfare is protected. Stress to the fish will be limited at all stages and analgesics will be administered to minimise pain following the procedure. The implantation and tagging procedures are thought to cause only transient pain or discomfort. Tagged fish will be released into the wild for behaviour monitoring and therefore not recovered. As such the device will remain with the fish for life. Due to the small size of the tag and its minimal weight it is unlikely to affect the long-term fitness of the fish or their natural behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The study will use wild-caught fish: for example, cod, bass, pollack, ballan wrasse and thornback ray. All fish used will be subjected to 'Moderate' severity.

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objective of the work is to examine the ecology and behaviour of wild fish in relation to changes in their natural aquatic environment through the development of offshore windfarms. The use of wild fish is necessary because the behaviour of hatchery-origin stock may be influenced by the condition and learned behaviour of these fish. Therefore, there is no viable alternative to the use of free-living, wild fish.

Which non-animal alternatives did you consider for use in this project?

We considered the following alternative approaches to replace the use of tagged live wild fish:

1) Baited remote underwater video analysis to video record the quantity and activity of fish in the sampling area.

2) Netting surveys. Netting surveys could provide some information about the presence or absence of fish at a given time and could be conducted over an extended period.

3) Catch-Tag, Release-recapture surveys.

Why were they not suitable?

There is no viable alternative to the use of wild live fish because we wish to establish how changes to natural marine environments by offshore engineering projects affect their choice of habitat and movements.

1) Using baited cameras risks affecting the natural behaviours and distributions of the target species and would provide only a 'snapshot' of activity in time. In addition, the sampling sites will periodically experience poor water visibility that could compromise this approach.

2) Netting options, including invisible gill-nets and trawling are indiscriminate (in terms of species caught and size of fish) and would damage or kill most of the fish caught. Moreover, netting would not provide detailed and continuous tracking information that would be obtained by telemetry.

3) Catch, Tag, Release and Recapture. Here, fish would be caught, tagged with an external, minimally invasive tag and then released into the wild again. If these tagged fish are later caught by sport or commercial fishermen the tag information would instruct them to report the location and date of recapture, thus giving information on the movement of the fish. The drawback here is that it requires very large numbers of fish to be tagged

because recapture rates are extremely low and cooperation from the fishermen is not guaranteed. The approach would not yield continuous movement data.

Our preferred (optimal) strategy is therefore, acoustic telemetry because it would cause least harm and suffering whilst producing detailed, long-term and continuous data on habitat use and movement of fish in and around the study site.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We carefully consulted existing peer-reviewed literature and other experts on the use of acoustic telemetry devices to determine the appropriate minimum number of fish required to obtain robust and meaningful outcomes.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The nature of this work (tracking the movement of mobile, free-ranging wild fish) is such that predictions of statistical power are not feasible. However, using the existing literature and after consultation with other experts in the field that currently use fish telemetry, we have established a minimum number of required individual fish that will be tagged to yield meaningful research outcomes. Fifty individuals of each species will be tagged and tracked (250 total). This will allow for fish lost through natural mortality, or to fisheries without compromising the project.

Our study site is an offshore windfarm (OWF) that allows us to carefully position our acoustic receiver units in a circular array, around turbine supports and with overlapping detection fields such that fish movement will be accurately pin-pointed and will not evade detection. Thus, the careful design of the receiver placement will ensure we can minimise the number of fish used. We anticipate that a number of will leave the detection site and, whilst some are likely to return, others may not.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Fish that are released into the wild with telemetry devices inside them will also be tagged with an externally visible marker ('Floy' tag) that request commercial fishing operations or sport fishers return them immediately to the water if they are incidentally caught (outside of this project). We have consulted with other experts using fish telemetry and established

that our chosen number of fish is a minimum that will yield robust and meaningful data. In addition, we will strategically place our telemetry receivers around the study site such that tagged fish within range will not evade detection.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

These species have been selected because of their economic value, behavioural characteristics, relatively large size and ease of capture. The size of the fish selected, relative to the telemetry devices implanted or attached, means that they will be minimally encumbered. Previous studies have shown that intraperitoneal devices of the kind we will use cause no adverse effects on fish including bass, provided they are sufficiently small relative to the size of the fish. We will use adult fish because of their larger size and because we wish to record data from sexually mature specimens. Using adult specimens will also ensure minimal handling due to their size during the procedure and therefore reduced stress.

Why can’t you use animals that are less sentient?

The research necessitates the use of sentient individuals because we are assessing the normal behaviour of adult fish in the wild.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Minimal fish handling at the study site and good fish husbandry and welfare techniques will minimise stress to the fish prior to and following the surgical procedure. We will assess the condition of fish and only use those deemed in good health for our study. Surgical implantation of acoustic telemetry tags into the peritoneal cavity ensures that the devices are not lost over the duration of the project. Previous studies have shown that fish recover rapidly from this implantation with no lasting harm, and we will minimise the burden on fish by using the smallest possible tags relative to the size of the fish. The use of an anaesthetic agent to sedate the fish during transmitter insertion and application of analgesia on pre and post -operatively will ensure distress, pain and suffering will be minimised. Before release back into the sea all fish operated on will be carefully assessed for recovery and condition and only those meeting or exceeding pre-operative condition will be liberated.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use online sources of information to stay informed about refinements. https://norecopa.no/ is an excellent resource for published works and best practices in animal welfare and research and contains an updated list of references relevant to a variety of species including fish.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly review our practices in consultation with peer-reviewed literature, and with reference to data sources including https://norecopa.no/ which is an excellent resource for up-to-date information on the use of fish in research and the 3R's. We will routinely monitor and utilise the NC3R's and Norecopa websites for updates and best practice aligned with the 3Rs. In addition, we will continually review our practices in consultation with the Named Veterinary Surgeon (NVS) and Named Animal Care and Welfare Officer (NACWO). These measures will be applied from the outset of the project in order to ensure any changes (reduction, replacement and refinement) can be applied in a timely manner.

93. Development of live bacterial therapeutics for the treatment of cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Microbiome, Cancer, Immunology, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify mixtures of beneficial, health-associated novel bacteria which have the potential to be used as bacteriotherapies to treat cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Currently around 1 in 2 people will develop cancer in their lifetime. The emergence of therapies that activate the immune system to fight cancer cells (immunotherapies) has led to increased survival for patients with many types of cancer. Unfortunately not all patients respond to these treatments. In these non-responder patients the particular bacteria within their gut has been found to impact their response.

This work will help us to understand the mechanisms whereby healthy gut bacteria species (microbiome) are able to enhance the immune response following treatment with immunotherapies. The research will aid in the development of new bacterial therapies to treat cancer.

What outputs do you think you will see at the end of this project?

The long-term output of this programme will be to identify the best bacterial therapies for the treatment of different types of cancer.

In the shorter-term this will involve making new discoveries on the types and behaviour of common gut bacteria found in people and how these interact with the immune system.

Discoveries made on new bacterial species will be published and available to other researchers.

Who or what will benefit from these outputs, and how?

The significant shorter-term output of the programme of work will be to generate data to demonstrate which bacterial cocktails can be used to progress to pre-clinical development in preparation for clinical studies in humans.

In the longer term, we expect our lead novel bacteriotherapies to increase the effectiveness of current cancer therapies such as immunotherapy in the treatment of lung cancer and types of skin cancer such as melanoma.

How will you look to maximise the outputs of this work?

The results of the research will be published in scientific journals and presented at scientific conferences. New techniques may be patented and shared with other researchers.

Publication of unsuccessful data or techniques will also be considered where possible.

Species and numbers of animals expected to be used

- Mice: 5500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice to grow tumours at the site where cancer cells are injected. Mice are widely used for this type of work, and have helped researchers understand how the body defence mechanisms react to tumour growth. Mice can therefore be used to predict the mechanisms of action and the effectiveness to treat disease of new medicines. Mice are also able to be bred without gut bacteria (germ-free) to enable us to measure the impact of bacterial therapies. All mice used will be adult mice as these will have a fully developed immune system.

Typically, what will be done to an animal used in your project?

The majority of mice on this project will be injected with cancer cells under the skin on the side of the animal. These cells grow as a solid tumour and are measured regularly to track lump size and mice are humanely killed before the maximum volume is reached. The gut of these mice will also be colonised with particular bacterial species either using samples obtained from human cancer patients or specific bacteria grown in the lab. Sometimes in order to help these bacteria grow we first need to give the mice antibiotics either in drinking water or by injection into the abdomen to eliminate the bacteria that already live in their digestive tract. At various times they will be treated with our candidate bacterial cocktails given by the mouth which requires putting a small tube into their throat for a very short period of time to deliver the bacteria into the stomach. We will use the least invasive way possible to administer the antibiotics, some of which can be in the food and drinking water but occasionally due to the type of antibiotic we need to do this by injection.

In general the bacterial cocktail will be administered orally under anaesthesia. A subset of these mice will be used in our therapy studies and will be administered with anti-cancer drugs by injection to the abdomen. These are treatments that are currently used in hospitals to treat cancer patients.

All these procedures will cause minimal suffering to the mice. All the mice will be check daily by qualified animal care staff.

A small number of mice who are cured of their cancer and the small lump is no longer detectable will be injected again with the same cancer cells under the skin to their opposite side. If cured of both tumours mice will be kept for observation for a maximum of 60 days. This may allow us to show that the effect on the immune system is long lasting and represents good candidates for long-term therapeutic benefit to cancer patients.

When the mice reach the endpoint of the study, defined by a humane endpoint such as tumour size or timepoint, they will be humanely killed prior to tissue collection. Some mice could have small blood samples collected while they are alive so that we can track the effect of a treatment and a further terminal blood sample under non-recovery anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice typically do not show any altered behaviour when administered cancer cells.

We select the cancer cells to be administered to be the most suitable for our experiment which give rise to tumours that are well tolerated. Very rarely the mice may scratch at their lumps causing the skin to be broken and if this is observed the mice will have their claws clipped to reduce the likelihood of damage.

Some of the immune system treatments, as they are designed to provoke the immune system, can give rise to clinical signs such as diarrhoea. This will be closely monitored (twice a day or more if necessary) and when it exceeds predefined thresholds the affected mice will be humanely killed.

Mice will be used to assess the impact of bacterial cocktails on the immune system of healthy mice without a tumour. These mice could experience some side effects from the treatments such as lethargy identified by a lack of responsiveness. All mice are carefully monitored and if they exceed pre-set observations such as weight loss, dehydration and changes to normal behaviour they will be humanely killed. We will provide extra bedding and additional support to the mice to minimise these effects.

Where mice may scratch at a tumour site, the preventative addition of trimming the claws of these mice will be implemented. This will allow them to relieve the itch but limits the damage to the site of the tumour.

Bacterial cocktails are administered orally under anaesthesia to reduce the movement of the animal and ensure all bacteria reaches the stomach. Thus reducing any likelihood of abrasion to the oesophagus and subsequent infection due to the bacteria.

The type of mice and experimental protocols that we are using are well established, widely used and designed to cause the least pain, suffering and distress to the mice.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice
- Mild approx. 8%
- Moderate approx. 92%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The immune system is extremely complex. During the development of cancer there is an interaction between the growing tumour and immune cells such as white blood cells. Immune responses directed towards growing tumours can kill tumour cells and eliminate cancer. These interactions are highly regulated and can not be replicated in experiments carried out in the laboratory.

Which non-animal alternatives did you consider for use in this project?

We are able to grow specific bacteria species from human gut within our laboratory. We then measure the basic interactions of these bacteria with immune cells, such as white blood cells, isolated from human blood samples. These non-animal alternatives allow us to determine the difference between good and bad gut bacteria without the need to use animals. We continuously consider their use and development in light of new experimental data.

Why were they not suitable?

It is not possible to replicate the full complexity of the interactions between bacteria and immune cells using laboratory experiments which do not use animals. Animals with an active immune system remain essential to understand these interactions under conditions within living tissues such as the colon and tumours.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Based on our extensive experience with tumour growth studies in mice, we use 8-10 mice per treatment group. This is a sufficient sample size to allow for the differences in tumour growth patterns we typically observe between individual mice. All experiments with a positive outcome are repeated at least once and a maximum of two times to ensure reproducibility.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We continuously evaluate our methods and experimental results to determine if we can further reduce the number of animals per experiment. We now believe that one of the major sources of variation is caused by the composition of the bacteria between mouse colonies and even between mice in different cages. The effect of mother-offspring proximity during the early stages of life has a strong influence of the composition of an individual's microbiota.

We will employ three measures to address this. First, we will inoculate germ-free animals with defined bacterial types (microbiota) or a purposely designed microbiota guided by our data. The second approach we will employ is to use littermates, who are genetically identical, such that the microbiota is controlled against cage to cage effects. A third approach is using wild type mice with a standard mouse microbiome that are treated to enable colonisation with human microbiome.

We believe these three approaches will lead to less variation between experiments and, as a result, the need for fewer animals to be used.

We also utilise the NC3R's experimental design tool (https://www.nc3rs.org.uk/experimental-design- assistant-eda) to aid in experimental planning and follow the most recent PREPARE Guidelines: https://norecopa.no/PREPARE to ensure all aspects are conducted to the highest standards.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be carried out on each tumour cell type to determine variability in growth. These data will aid in optimising the numbers of animals required per group to effectively determine any impact of treatment on tumour growth.

Wherever possible, multiple experiments will be performed on the tissues collected from an individual mouse, including harvesting mouse tissues for experiments or cells that can be frozen and stored. For example, the spleen can be used to isolate mouse immune cells for further assays. Tumours can be harvested for analysis of the immune cells within and DNA collected to measure changes in mouse genes with treatment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For this project our idea is that defined bacteriotherapies either alone or in combination with anti-cancer drugs can block or cure cancer. We used peer reviewed literature to guide our tumour type selection alongside in-house data.

This project will use well-defined and optimised tumour types, such as melanoma, grown in mice with no gut bacteria (germ-free) and specified known bacteria (gnotobiotic). To assess the impact of the host microbiome mice can bred and maintained germ-free allowing us to inoculate animals with defined bacterial cocktails so we can monitor the activity of the host and microbiota with sophisticated molecular approaches.

Why can’t you use animals that are less sentient?

Mice are used in this project as they share many of the same species of gut bacteria as humans. Animals with a more immature life stage stage do not contain these same bacteria and are not as easily bred and maintained in a germ-free 'bubble' within isolators.

The mice are required to carry out normal habits such as eating and drinking to enable us to measure the impact of orally ingested substances on the gut bacteria.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Germ-free mice are bred and housed in cages within 'bubbles' or large contained isolators which prevent the entry of viruses or bacteria. The cages contain environmental enrichment and mice are housed within social groups of 4/5 animals.

Mice will have two daily welfare checks which include observing that animals are healthy and can move freely in every cage, have sufficient food and water and the isolator temperature/humidity readings are appropriate. These details are recorded on observation sheets within a day book for assessment of an individual animal's health status.

Where novel agents are administered, the duration and frequency of monitoring including periods of continuous monitoring will be increased for example, following administration of novel agents. A single animal will be dosed and monitored for one hour before the other animals on study are dosed.

For mice which receive oral administration of a mammalian microbiome, this procedure is carried out under anaesthetic to reduce the likelihood of any harm due to movement of the mouse during the process and post procedure checks are carried out. We will continue to work with animal care staff to assess the requirement for anaesthetic use.

Active use of and continuous development of humane endpoints will allow us to continually refine procedures. For example in the measurement of tumour size we are considering

trialling the use of non- invasive methods such as Biovolume http://www/biovolume.com in comparison to standard calipers.

Currently once a tumour reaches 700mm3 it is flagged as yellow and will subsequently be measured daily.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure best practices we follow the updated ARRIVE guidelines [PLoS Biol 2020 18(7): e3000410]

We also follow the PREPARE guidelines to ensure all best practices are followed by researchers who use animals on this licence. [Lab Anim. 2018 Apr;52(2):135-141. doi: 10.1177/0023677217724823.

Epub 2017 Aug 3.]

In addition, we follow updates to best practice guidelines on The Laboratory Animal Science Association, LASA and NC3Rs websites.

We will publish to ARRIVE and PREPARE guidelines and where possible data will be presented at scientific conferences.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will use the NC3Rs website to gather up to date information about advances in animal research. This information will be disseminated to anyone involved with this licence including Project Licence Holder (PPLH), Personal Licence Holders (PILs), Named Animal Care and Welfare Officers (NACWOs), Named Information Officer (NIO) and Named Veterinary Surgeon (NVS) and implemented where appropriate.

94. The role of nitrite in coronary artery disease and its associated risk factors

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Coronary artery disease, Hypertension, Diabetes, Mechanisms, Therapy

Animal Types	Life Stages
Mice	adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project investigates nitrite as a therapeutic target to improve the poor heart and blood vessel function associated with diabetes and high blood pressure, which are major contributing factors to the development of coronary artery disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work? What is the role of the heart and blood vessels?

The heart pumps blood through blood vessels, which carry the blood to and from all areas of the body. This whole network of vessels for blood circulation is called the vascular system.

The heart receives its own blood supply through blood vessels known as coronary arteries, which like all other tissues in the body, needs oxygen and nutrient-rich blood to function. If these blood vessels are damaged or diseased, this gives rise to coronary artery disease (CAD; sometimes known as coronary heart disease).

What is heart attack?

CAD is the most common type of heart disease, and the first sign of this is a heart attack (myocardial infarction). This happens when there is a narrowing or blockage of a blood vessel, which prevents blood flow to the heart. In the UK, there are approximately 63,000 deaths due to heart attacks per year.

How does diabetes and high blood pressure increase the risk of developing a heart attack and increased heart injury?

There are several chronic conditions (also termed comorbidities) that increase the risk of CAD development, including diabetes and high blood pressure (hypertension). Diabetes is caused by high blood sugar levels for a prolonged period of time, which can lead to the lining of the blood vessels becoming thicker and therefore restrict blood flow and cause serious heart complications. High blood pressure can cause significant strain to the blood vessels, which subsequently become damaged and make it harder for the blood to flow around the body.

Why is it important to undertake this research?

It is estimated in the UK that 3.9 million people are diagnosed with diabetes and 12.5 million people are diagnosed with high blood pressure, figures which are increasing both nationally and globally. Studies have revealed that patients with diabetes and/or high blood pressure are more at risk of having a heart attack. Furthermore, patients with these comorbidities tend to experience increased adverse effects by causing worsened heart cell death following heart attack. This can affect clinical outcomes profoundly by increasing mortality.

There is an urgent need for effective therapies that prevent blood vessel and heart abnormalities from these comorbidities and therefore limits heart injury when a patient has a heart attack.

Therefore, understanding the underlying mechanism of diabetes and high blood pressure is essential, and how to prevent further heart cell death following heart attack in the presence of these comorbidities is warranted.

Nitrite as a potential treatment against cardiovascular (heart & blood vessel) disease

Currently, there are very few treatments available to protect against blood vessel and heart abnormalities caused by diabetes and/or high blood pressure. Our research group is investigating a chemical called nitrite as a potential treatment strategy for improving blood vessel and heart function.

Nitrite is found in green leafy vegetables (e.g. spinach, beetroot) and work carried out under our previous licence we discovered a new mechanism in how it is able to regulate blood vessel and heart function during normal, healthy conditions. However, we do not know how nitrite functions during periods of ill-health - such as diabetes and high blood pressure - and whether use of nitrite would prevent further heart injury following a heart attack under these comorbidities.

Therefore, the work to be carried out under this licence is essential to:

• Identify key signalling pathways of nitrite in how it modulates blood vessel and heart function, which will further scientific knowledge

• Advance the understanding in how nitrite works on blood vessel and heart function during diabetes and high blood pressure

• Identify the key targets of nitrite mechanism that could lead to the development of new therapies to treat against blood vessel and heart abnormalities observed in diabetes and high blood pressure

• Assess whether nitrite has the ability to reduce heart tissue injury following a heart attack in the presence of diabetes or high blood pressure

What outputs do you think you will see at the end of this project?

The ultimate aim of this licence is to better understand the role of nitrite on blood vessel and heart function when they become diseased (e.g. diabetes or high blood pressure) and whether it can reduce heart tissue injury following a heart attack (myocardial infarction). This new information will enable us to identify potential new treatment targets that could be used to prevent or slow the progression of heart disease. These treatments could lead to the generation of new medicines with significant subsequent improvements to human health.

Our primary outputs will be in the form of publications describing these mechanisms in scientific journals and conference communications. We hope that these publications will be useful for other researchers and paves the way for new research, ultimately translating our findings to patients at risk of heart disease.

Who or what will benefit from these outputs, and how?

Short term: Gain an understanding in the underlying mechanisms of healthy and diseased blood vessel function and the development of a heart attack. Our colleagues and collaborators will benefit from the data that will inform them their experiments and approaches.

Medium term: The mechanisms and pathways identified will enable us to target specific molecules/proteins with potential to aid development of novel therapies. Our colleagues and collaborators will benefit from the data that will inform them their experiments and approaches.

Longer term: We anticipate that we will advance our understanding of the mechanisms underlying blood vessel dysfunction and heart attack. This project may identify key signalling pathways that could lead to improvements in current therapies and development of new therapeutic targets for heart disease associated with blood vessel dysfunction. All new findings will be shared with the wider scientific community, published in journals and scientific conferences.

How will you look to maximise the outputs of this work?

To maximise the output from this work, we are collaborating with chemists, biochemists, pharmacologists and clinicians nationally and internationally. The findings from this project will be presented at conferences and published in peer-reviewed original research articles. In the longer-term, we will engage with fundraising committees and patient groups to discuss our key findings for new drug treatment. To enable wider dissemination (local, national and international exhibitions) we will develop an interactive stand to illustrate the concept of our research in cardiovascular disease. A proactive media engagement strategy will be implemented to raise public awareness of cardiovascular disease.

Species and numbers of animals expected to be used

- Mice: 6500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Blood vessel dysfunction and how it progresses in the development of heart attack are complex multifactorial syndromes which involve the interaction of numerous body systems and various factors (e.g. high blood pressure, diabetes) over a prolonged period of time. As such, it is impossible to evaluate the mechanism and novel drug targets in a single cell isolated set-up.

In this project, we will use adult mice as their cardiovascular systems are fully developed and are similar to human cardiovascular function and molecular pathways.

We choose to use mice as this allows us to breed and use mice which are genetically modified, thus allowing the genome of the animal to be manipulated so that the protein product of a particular gene is not produced (or in some cases the manipulation can be so that more of the protein is produced). Using genetically altered mice will help us to better understand the human disease and identify new treatments.

Furthermore, we will be able to draw on a large body of existing knowledge for this species to allow rapid progress and large number of well-established procedures are available to model key aspects of human disease.

Typically, what will be done to an animal used in your project?

To identify the key signalling pathways of nitrite, we will use normal and genetically modified animals which lack genes specific to important pathways regulating blood vessel and heart function.

In these set of experiments, animals will undergo non-recovery terminal anaesthesia under which tissues will be removed for further analysis on specialised laboratory equipment for the assessment of blood vessel function and/or models that mimics heart attack.To evaluate the mechanism of nitrite in diabetes, we will use diabetic mice. In these set of experiments, blood sampling will be taken from the diabetic animals to monitor blood sugar levels and may be administered with nitrite or control (e.g inactive treatment control group). Similar to above, the animals will undergo non-recovery anaesthesia for the removal of tissues for analysis in the laboratory.

In this project, we will also evaluate the impact of high blood pressure on blood vessel and heart function. We will use normal and genetically modified animals which lack genes specific to important pathways related to regulating blood pressure. Some animals will be administered with pharmacological agents via previously implanted subcutaneous mini-pumps and may also receive an injection of control (vehicle) or nitrite. Mice will have their blood pressure measurements taken using standard tail cuff (non-invasive) method on several occasions before and after medication or no treatment (control). Tissues will again be taken under non-recovery anaesthesia for further analysis in the laboratory.

For all experiments, once tissues have been taken under non-recovery anaesthesia, they will be used on a specialised laboratory equipment for the assessment of blood vessel function and/or used in models that mimic a heart attack.

What are the expected impacts and/or adverse effects for the animals during your project?

The proposed mouse models of diabetes and high blood pressure models are well-established locally.

Some mice will have an osmotic pump implanted to induce high blood pressure whilst they are anaesthetised and this may cause some temporary discomfort. Mice will be monitored

daily for their general well-being and weighed on weekly basis. Should animals show signs of distress they will be killed humanely.

In some protocols, blood pressure will be measured non-invasively (like in humans with a blood pressure cuff). It is unlikely that this will result in harm or distress; all animals will be handled using non- stressful handling techniques and application of the device itself does not cause pain.

Some animals may experience mild discomfort from receiving an injection. Some mice may receive multiple drug injections or undergo repeat blood sampling (for example in diabetic animals to measure blood sugar levels). If this is required, the routes and volumes will be suitable for their age and size and will be such that animals fully recover between interventions and will not suffer more than transient pain, distress and/or long-lasting harm, and we will make every effort to minimise cumulative adverse effects.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Non-recovery 50%
Mild 25%
Moderate 25%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The events that occur in the development of heart disease are complex, involving changes in blood flow, oxygen supply to tissues, and inflammation. Therefore, it is not possible to measure tissue injury in cell culture models as there is disproportionately larger extracellular space (e.g. the culture medium), that prevents the accumulation of metabolites and the profound lack of oxygen (hypoxia) and substrate deprivation that accompanies ischaemia in a whole heart model. Consequently, findings in cell-based models of simulated ischaemia always need to be replicated in an in vitro whole heart model.

Therefore, animals must be used when measuring the consequences of ischaemia on the remodelling process. This process is complex and results from changes in all the cell types within the heart, which in turn are modulated by circulating agents, such as neurohormones and inflammatory mediator, as well as circulating cells. The processes cannot, therefore, be modelled in a cell culture.

Which non-animal alternatives did you consider for use in this project?

We are using isolated organ applications for testing the blood vessel (myography) and the heart (Langendorff preparation). For the assessment of processes at molecular level we have considered using commercially available cell lines or cells from relevant mice. When feasible, we use obtain clinical samples, such as blood vessels and heart biopsies from patients undergoing heart surgery, in order to minimise and reduce the number of animals required.

Why were they not suitable?

Certain mechanisms that will be studied in this project using animals cannot be studied in cell culture due to the complexity of the cardiovascular system and uncertainties/unknowns in these diseases.

Human tissue samples are difficult to obtain and the information we can obtain from human tissue samples is limited because of the legal constraints on access of tissue, and ethical constraints on experimental interventions in patients for the treatment and diagnosis of disease. Therefore, due to these circumstances we are unable to do all our research in isolated cells and humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals to be used in this project based on past experience and relevant published work. We have performed statistical analysis to work out the number of animals we will use for this project. The proposed experimental design and methods of analysis of the results have also been reviewed by a statistician.

Importantly, when estimating total numbers, we have taken into account the fact that some animals might serve multiple purposes (e.g organs and tissues used for different objectives). Therefore, all experiments have been designed to minimise the number of mice needed to make statistically meaningful conclusions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our breeding colonies for the genetically altered animals are tightly monitored to ensure that we have sufficient size to produce the number of animals needed without significant excess. Whenever possible, tissues from the same animal will be used for multiple purposes and will be shared amongst team members working on this project (e.g. heart, blood vessel, blood samples etc…), thus reducing the number of animals used. In addition, serial assessments (e.g blood pressure, blood sampling) to reduce experimental variability by allowing comparison at different time points in the same animal, therefore reducing number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For each and every experiment, as part of good laboratory practice, we will start experiments with a small pilot of a few animals to ensure that there will be no unexpected welfare harms and assess whether if it is needed to go on to a large experiment with more animals. We will harvest multiple organs and tissue and share them amongst the research group to perform experiments in the laboratory to minimise the number of animals is used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Currently the mouse cardiovascular system is considered a robust model for that found in humans and has the advantage of genetic manipulation. The genetically altered strains are lacking important mechanistic elements which helps us to understand the mechanisms of blood vessel and heart function. We are already breeding these genetically altered mice under a different licence and the mice do not show any clinical signs or suffering.

We have also designed this project to avoid suffering and distress in the mice:

1. We harvest the heart and associated blood vessels under non-recovery anaesthesia and use ex vivo techniques to measure blood vessel and heart function. This is a major refinement to our protocols as we are not using procedures in living animals to assess blood vessel and heart function, which minimises severity and suffering.

2. The models of high-blood pressure and diabetes we are proposing to use in this project are well- established. Therefore, we have good knowledge of the course of the pathology and so can refine our endpoints.

3. We will use non-invasive techniques to measure blood pressure, which is the most refined method available and minimises suffering to the animals.

4. Several steps will be taken to minimise stress resulting from administration of medication. For example, the use of refined handling methods to prevent distress and minimizing injection frequency to prevent lasting harm.

Why can't you use animals that are less sentient?

Species that are less sentient do not share common characteristics with humans nor are they sufficiently characterised to be reliably used as models of human disease. Animals are used at the earliest of their life stage, but the composition of the scaffold of blood vessels and heart changes, builds up over life, and we can only use mice which have fully matured blood vessels and a developed heart (e.g. at 8 weeks of age).

Control of blood pressure is significantly affected by general anaesthesia. Therefore, whenever possible, and welfare concerns can be addressed, physiological measurements will be performed in conscious animals. It is important that our scientific research is transferable, where the results have relevance to the cardiovascular disease in humans.

Tissue and blood vessel isolation is performed in mice which have been terminally anaesthetised.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All the experiments undertaken in this project are designed to use procedures that cause the least pain, suffering and lasting harm. The major refinement to our protocols is in the area of harvesting tissues under non-recovery anaesthesia, which minimises suffering in the mice. In addition, when anaesthesia is applied, the efficacy is monitored throughout the procedure.

Some animals will be administered with a pharmacological agent and may experience mild transient discomfort from receiving an injection. If blood sampling is required, routes and volumes will be suitable for their age and size and will be such that the animals recover between interventions and will not suffer more than transient pain and distress. This will ensure there is no lasting harm and therefore we can minimise cumulative adverse effects.

Administration of pharmacological agent is performed according to the route and dose that minimises adverse effects and input will be encouraged from the animal technicians and Named Animal Care & Welfare Office (NACWO). Since stress and anxiety affect the control of blood pressure, all animals will be handled using non-stressful handling

techniques (such as tunnel handling). They will also be allowed to adapt to experimental conditions (e.g. measurements of blood pressure via tail cuff) in advance of experiments to reduce anxiety and stress. All animals will be closely monitored throughout the study.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We actively check for up-to-date publications relevant to the techniques used in this project to ensure that we are conducting experiments in most refined way and to maximise scientific output. Major guidance through the project is taken from the NC3Rs, whose website (https://www.nc3Rs.org.uk/3rs- resources) provides resources on the general principles underlying the experiments highlighted in this project. This includes anaesthesia, breeding strategy and numbers, and experimental design.

LASA guidelines will be used for administration of substances, volumes and frequency limits with the most up-to-date veterinary knowledge (https://www.lasa.co.uk/current_publications/)

We will publish according to ARRIVE guidelines 2.0 (https://arriveguidelines.org) to help ensure that experiments are reproducible with minimal wastage of animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular updates from organisation (e.g. newsletter) and the NC3Rs website. In addition, we have regular discussions and updates with the staff in the animal facility to ensure that we are using up-to-date information in relation to the 3Rs. Regular updates are also sourced from attending scientific conferences and publications from cardiovascular journals.

95. Modulating the immune response in cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunomodulation

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify novel mechanisms by which substances that modify immune responses (immunomodulatory substances) can initiate, enhance or maintain anti-tumour immune responses in vivo. These studies will not only provide fundamental understanding of the requirements for an efficient anti-tumour immune response, but should also provide proof of principle data for future therapeutic development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is the second leading cause of death worldwide, accounting for 9.6 million deaths every year. However, new therapies targeting pathways known to inhibit the immune system have revolutionised cancer therapy, and clearly demonstrated the importance of the immune system in tackling cancer.

Unfortunately, a large number of cancer patients are non-responsive to such immunotherapies, highlighting the incomplete understanding of the mechanisms underlying the activation and suppression of the anti-tumour immune response. Therefore, it is critical to gain a greater understanding of the processes leading to a successful immune response during cancer and to identify novel targets and immunomodulatory substances that could be used alone, or in conjunction with current therapeutics, to improve cancer treatment.

What outputs do you think you will see at the end of this project?

A central goal of this project is to identify new understanding of the mechanisms by which immunomodulatory substances can initiate, enhance or maintain anti-tumour immunity.

One of the immunomodulatory substances that we will initially focus on (IMM-101) has already been tested in phase I and II clinical trials. As part of this project, we aim to generate vital preclinical data to help this promising therapy to advance to phase III trials and, ultimately, to the clinic.

This project aims to disseminate new knowledge by publication in peer reviewed journals and presentations at conferences, seminars and workshops. We hope that in the longer-term our work will contribute to new immunology-based therapies for cancer. In the 5 years of this project, we aim to continue our high standard of publication, averaging more than 5 research papers per year in highly- respected peer-reviewed journals.

Who or what will benefit from these outputs, and how?

This project aims to answer basic scientific questions. The new knowledge generated, and the unravelling of important fundamental immune mechanisms, will be relevant to a broad range of human and animal conditions, including cancer.

In the short term, we will gain novel mechanistic insights into how the immune system can be modulated to promote tumour clearance. This new information will offer the potential of enhancing future treatment options for a broad range of disease, as the immune response is centrally involved in all cancers. Importantly, one of the immunomodulatory substances that we will initially focus on (IMM-101) has already been tested in phase I and II clinical trials, and has demonstrated safety and efficacy in advanced pancreatic cancer and melanoma, and is currently being assessed in a range of other cancers. Therefore, increased mechanistic understanding of this promising therapy is critical for its progress to phase III trials, and to patients.

In the longer term, we aim to discover novel therapeutic candidates through our fundamental research into anti-tumour immune responses. These novel candidates could be targeted individually, or combined with established therapeutics, to improve current cancer treatment strategies.

How will you look to maximise the outputs of this work?

Communication of our findings will be primarily through publication in widely-read peer-reviewed journals, but also presentation at local, national and international congresses and institute seminars. To ensure maximum dissemination, only journals that allow open access without payment by the reader will be considered. To prevent unnecessary repetition of experiments by others, we will seek to publish all data generated under this project, including negative results.

To enable rapid translation of our findings to the clinic we will exploit new and existing collaborations, and with local clinicians as part of the translational environment within our institution. We have highly effective systems in place for technology transfer. Additionally, we aim to expand our current collaborations with pharmaceutical and biotech companies by presentation at national and international forums at which industrial representatives are present.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We study adult mice because the immune system, tissue organisation and development of all mammals are similar, allowing mice to be a model for humans and other animals. We also use mice because scientists have created many genetically altered mouse lines that allow us to dissect in fine detail what happens during immune responses and in cancer. Genetically altered mice, and many of the tools designed to work with mice, allow us to define in precise detail how particular cells and molecules of the immune system work together to fight cancer. By manipulating these cells and molecules, we can identify the immune components that are most effective at preventing tumour growth, and use that information to help design future cancer therapies.

Typically, what will be done to an animal used in your project?

Typically, animals will receive a single or multiple injections containing an immunomodulatory substance (e.g. antibodies to neutralise a specific immune mediator or deplete a specific cell type, or cells to promote a particular response) and/or cancer cells.

Experiments might look at the immediate immune response in the first few days after administration of an immunomodulatory substance or tumour cells, or may last several months to allow full tumour development, or assessment of immune memory. These experiments will typically last between 1 and 28 days. For longer experiments, mice are likely to receive multiple doses of an immunomodulatory substance (e.g. once a week for 2-3 weeks). For some experiments, mice will receive an injection of tumour cells either prior to or after treatment with immunomodulatory substances. Depending on the substance, it may require multiple doses over a time period as tumours develop (typically up to 4 weeks). Experiments will end with animals being killed humanely, sometimes under terminal anaesthesia.

The cumulative experience of mice will typically be exposure to 2 or 3 procedures that may each cause short but usually separated periods of typically mild or potentially moderate degrees of suffering.

Separate from the above experiments, some genetically altered animals will be used only to breed and maintain animal lines.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals will experience no adverse effects or only mild adverse effects.

Tumours grown on the flank can affect walking and normal behaviour and, in rare cases, can become ulcerated. However, the tumour models we will use are generally well tolerated and will rarely reach moderate severity. Although immune modulation can trigger systemic inflammation that can cause weight loss, piloerection, hunching and reduced movement, in most cases these effects should be mild or transient.

In all experiments, animals will carefully monitored and humanely killed before they exceed moderate severity limits.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect approximately 85% of mice to experience mild severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mammalian immune system is highly complex, relying on the co-ordinated actions of multiple different cell types and molecules that collectively provide protection. As such, the insight in vivo experiments can provide on the mechanisms necessary for tumour protection is of significant clinical relevance. Importantly, we know the importance of the tumour microenvironment and its ability to suppress immune responses, thus limiting the efficacy of immunotherapies.

Unfortunately, in vitro systems are unable to reflect the cellular and molecular complexity of the immune system and tumour microenvironment. Therefore, the use of mammals is essential for gaining a better understanding of the mechanisms underlying immune protection that could be utilised for patient benefit.

Mice will be used in these studies because their immune system closely resembles the human immune system therefore giving a better chance for translating potential therapies. Additionally, a wide array of wild type and genetically altered strains of mice are available that will allow us to better decipher the role of immune cells and molecules in anti-tumour immunity. Finally, a vast range of reagents is available for analysing murine cellular and molecular interactions during immune responses.

Which non-animal alternatives did you consider for use in this project?

1) Analysis of human biopsies.

2) Use of cell lines and in vitro systems.

Where possible, we will use in vitro assays to provide initial data on the effects of immunomodulatory substances on specific immune cells. This data will then be used to inform and complement our in vivo experiments.

Why were they not suitable?

The types of experiments required to track cell function in vivo are not possible with human tissue biopsies, nor can we experimentally manipulate humans.

Many location-specific features of cells are lost once they are removed from the tissue, which makes the use of cell lines impractical. In vitro systems typically allow for the study of one or two cell types in a highly controlled environment that is not reflective of the complex immune system in vivo. Therefore, to fully understand how different cell types and molecules co-ordinate an effective immune response we require in vivo experiments.

We will regularly review the literature regarding in vitro and zebrafish systems so that if new approaches are developed we can test them and potentially exploit them if they succeed.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice has been estimated based on experience gained under my previous Home Office licenses, taking into account breeding strategies for genetically altered mice, and anticipated numbers of planned studies over the course of the license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For all of our experiments, in-bred mice are used to reduce experimental variation, which makes it possible to use fewer animals to achieve statistical significance. For the majority of our studies, mice from the same litters are used for control and experimental mice, reducing variation that can occur due to differences in the microbiota. Overall, our experiments are designed to reduce the number of variables (for example age) to as few as possible and thereby reduce the number of control groups required.

We work with the NC3Rs Regional Programme Manger to ensure all lab members are introduced to the NC3Rs experimental design assistant and encouraged to use it. Everyone in the lab is trained in statistical methods and these are regularly discussed at lab meeting, to ensure all agree the best methods are being used. This includes randomisation and blinding, whenever practically possible.

Tissue-sharing is a major tool we use to reduce animal usage.

A significant proportion of our animal use is related to breeding programmes for genetically altered lines. We follow the advice of our animal facility staff to optimise breeding, and regularly discuss numbers at lab meeting to ensure we do not overbreed. Where possible and appropriate, we use substances that can target or block immune processes in wild type mice, to reduce use of genetically altered mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We routinely perform pilot experiments to determine the optimal number of mice to achieve statistical power. Experiments are then performed on a minimum of two separate occasions to ensure reproducibility, following which data pooled from experiments are statistically analysed to reveal less pronounced effects without increasing overall animal use.

We have many years of experience in planning animal experiments and we plan our research to ensure that all animals are used most effectively. We often combine experiments to ensure that multiple organs are used to address multiple objectives at once. Careful discussion between multiple researchers is required to avoid compromises. Due to high variability in immunological and tumour models in vivo, especially in genetically altered models that we have yet to assess, we will adjust groups sizes as required, should subsequent power calculations indicate that this is necessary.

The increased use of genetically altered animals has led to more complicated breeding strategies and, as a result, larger colonies. We reduce the numbers of these animals in our experiments by using littermates as controls wherever possible. Additionally, when a particular strain is not being used experimentally we work closely with the animal technicians to develop a breeding strategy that maintains low numbers of stock animals.

In many experiments, we will use bone marrow to carry out pilot studies in vitro, therefore only a few animals are used to initially test new hypotheses. Importantly, we continue to work closely with collaborators who can supply bone marrow samples, therefore reducing the number of genetically altered animals that have to bred to facilitate experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mouse models to study the immune response in cancer. Mice represent the most appropriate species for in vivo study of cancer immunity, because of the extensive knowledge of their physiology as it relates to humans, the genetic and biological tools available and the ability to be easily bred and handled.

The cancer models we will use do not cause significant pathology, and doses and timing are carefully managed such that the animals will experience minimal suffering. As our experience of these tumour models develops, we will look to refine our approaches to ensure robust experimental results whilst minimising pain, suffering or distress.

Why can't you use animals that are less sentient?

To our knowledge, no other species of lesser sentience can fulfil the requirements of this project to the same extent as the laboratory mouse. We are studying long and complex immune processes, and trying to understand how the adult immune system handles tumours, and how different cells communicate to orchestrate an appropriate response. Only adult animals would give meaningful results. To define the impact of immunomodulation on tumour growth requires mice to be monitored for several weeks, so procedures cannot be carried out on terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All procedures will be performed by trained and skilled personal licence holders, who will handle animals with care. Animals will be monitored for adverse effects using score sheets previously developed in conjunction with the NVS and NACWO. These score sheets have proven to allow for objective measurements of clinical signs associated with adverse effects to determine when humane endpoints have been reached.

In line with the establishment's policy, we will adopt the latest techniques in animal handling (e.g. cupping) to significantly reduce the stress associated with procedures. Furthermore, where possible, the least invasive methods for dosing and sampling will be applied.

Anaesthesia and analgesia will be provided where suitable (e.g. for humane restraint, during or in recovery from surgery). The best aseptic technique will be used during surgery.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow LASA guidelines, and consult the recommended https://www.nc3rs.org.uk/3rs-resources on a regular basis, including watching videos of best practice techniques. For specific models, we read papers from other groups doing similar experiments, as well as consulting directly with other researchers to discuss the most refined procedures.

We will continue to use the NC3Rs Experimental Design Assistant to ensure we design experiments that will allow us to achieve statistical significance whilst minimising the number of animals we need to use.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our animal facility includes a team of dedicated veterinarians that are continually seeking to improve animal welfare and refine animal use. We consult closely with them and take full advantage of the extensive resources provided to ensure we are following current best practices. We will continue to work closely with our local NC3Rs representative to ensure we stay informed about the advances in the 3Rs. For example, we have recently attended an NC3Rs Experimental Design Assistant workshop to ensure we are using the EDA to the fullest extent in order to achieve our scientific aims with the minimal number of mice.

96. Trans-generational effects of age in birds

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Evolution of ageing, Ornithology, Trans-generational effects, Epigenetic

Animal types	Life stages
House sparrows	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand how age influences how inheritance can occur via non- genetic, yet heritable, mechanisms. I will address this knowledge gap by testing the hypotheses that (1) an organisms' age does not only affect its own traits, but also those of its progeny produced at different ages. Any such non-genetic, yet heritable mechanism is called epigenetic. We also hypothesize that (2) such age-associated effects are carried over into future generations, under wild conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Trans-generational effects can influence how selection acts and traits evolve. In fact, ageing can induce change to the DNA or even change what is in an ejaculate. These changes then influence the behaviour, physiology, and ability to reproduce of future

generations. However, while we know a little about these mechanisms in laboratory animals, we do not know how often they occur, and what their significance is, in wild animal populations. Notably, we do not know how, and how much these epigenetic modifications affect the ability to reproduce. Furthermore, past experiments been unable to distinguish between the different influences of genetics, the environment, and the epigenetic effects of parental age. This is mainly due to the difficulty to conduct in-vivo fertilisations in wild animals. Yet this knowledge is crucial to understanding the role of trans-generational effects, and ageing, in evolution, ecology, biomedicine, gerontology, and other fields. My long-term data from a wild bird population, together with a cross-foster design, can overcome this problem. A better understanding of the trans- generational effects of age will significantly help explain how longevity can evolve, and may guide the way towards a healthy, long life.

What outputs do you think you will see at the end of this project?

My project involves contributions to two long-term animal studies that are going on for decades now. These so-called long-term population studies are rare due to the logistic challenges of keeping long- term projects going for so long. However, these data are also crucial, and central for our understanding of ageing and longevity in wild populations, and evolution in the wild.

As such, I anticipate that the data collected under this project license will lead to at the least 10 major publications, and several minor ones, during the duration of the project license. As this data goes into our long-term database, the likelihood of more outputs after the duration of the project license is close to 100%. Thanks to the nature of the longitudinal data, I anticipate that these data will contribute to publications throughout the next decade and beyond.

The data from the wild will give us unprecedented insight in the ageing process of natural populations the so-far elusive trans-generational effects of ageing. There is currently keen interest in each of the individual aspects of the proposed study, in particular trans-generational effects, and the epigenetic mechanism. Only a "natural laboratory" of the type I use, where we can collect data and samples over a long period of time allow these questions to be pursued in a realistic framework.

Furthermore, our work routinely now contributes to an online database that is (on request via a dedicated website) openly shared with other reserachers in the field. The data from this project will contribute to this database, and as such reduce the need for similar data to be collected in other projects.

Who or what will benefit from these outputs, and how?

The nature of this rare data on a long-term population, recent advances in statistics, theory, molecular and genetic methods, mean that I expect to answer several major, longstanding questions that are likely to be of wide interest, both to the research community and to the wider public. Among other end-users, the results will be of most

immediate significance to conservation biologists who require the tools to manage the consequences of human induced global change. Furthermore, the results will also add significantly to the body of knowledge on the house sparrows, a species that, though still numerous, has been declining rapidly and without explanation in recent years in the UK and many other European countries. The results should also contribute significantly to our understanding of the evolution and maintenance of the differences in ageing rates, which is a big question in biology, in gerontology, reproductive biology, and even in sociology and politics. This study will improve our understanding of the evolution of ageing.

How will you look to maximise the outputs of this work?

To maximize the benefits from this work I will disseminate the results both through scientific publications and presentations and, where appropriate, the media and direct contact with the public. I have a good track record of publishing my work in high impact journals, including open-access journals, and of disseminating the results more widely, especially through press releases and subsequent interactions with the international media. I will publish our results in open access journals, or in journals with the gold standard open access policies. People who will be involved on this project will present the results at conferences, seminars, and workshops. We regularly hold outreach events.

Species and numbers of animals expected to be used

- Other birds: No answer provided

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying animal behaviour, ecology, and evolution in wild animals. There is no replacement for studying the live and wild animals when we want to know their behaviour, reproduction and genetics, and their response to occurring environmental changes. This is very different from medical research where one is interested in a certain disease and uses a model system to study the disease. We study wild animal behaviour and per definition thus need to study wild animal behaviour.

We study house sparrows because they are gregarious birds, that easily accept nest boxes and thus are easy to study. A similar argument is made for blue tits. Both are central model species in the fields of ecology and ornithology (maybe similar to mouse and rat models in medicine).

There is a large knowledge gap especially in our understanding of ageing and trans-generational effects of age between findings in laboratory animals, and those in the wild. For a long time, it was thought that wild animals do not show signs of old age, but among others, my past research has proven this wrong.

To understand the evolution of trans-generational age effects, it is necessary to observe wild animals age, because firstly, captive laboratory animals are typically bred to reduce variance, meaning there is a reduced genetic diversity. This reduced genetic diversity will affect how individuals age, especially as e.g., mice or rats are typically bred to only live for a short time, thus unknowingly, in such strains, any senescence effects will not have been considered during selection processes. Furthermore, to understand how much mortality comes from catastrophic and environmental effects, and how much is due to senescence requires longitudinal sampling in wild animals, as the former does not take place in captive animals. Next, due to the lack of variable selection pressures we cannot use results from the laboratory to interpolate to the wild, requiring the use of wild populations to really understand the evolution of ageing.

Yet, using wild animals in the study of ageing is difficult because it requires longitudinal data - that is data on the same individuals sampled at multiple times throughout their lives. This allows one to distinguish effects of age that change within-individuals, from those effects among individuals. For instance, imagine a population of animals where young ones that are also small die at a higher rate than young individuals that are large. Any observer who would not know of this fact, and sampled cross- sectional (meaning, measuring individual's age and size only once each) would think that older individuals are larger than younger individuals, because none of the smaller ones live long. Then one would draw the wrong conclusion that individuals grow larger as they age. To avoid this pitfall in ageing research, one must sample cross-sectionally - sampling individuals repeatedly over time - termed longitudinal sampling (Fig. 1).

However, longitudinal sampling, thus repeated capturing, is exceptionally difficult to achieve, because wild animals are often highly mobile and might enter or leave a study area unbeknownst to the researcher. This is the reason we know so little about ageing in wild populations.

In this project, I will make use of two populations of birds well suited to overcome these problems. One of these populations of birds lives on an island far enough away from the mainland, for the birds to be unable to fly across such large stretches of water. As such, we can be confident that when we do not encounter a bird repeatedly, that it has died from natural causes. Furthermore, the island population is small enough that we can monitor all broods, mark all chicks that hatched on the island, and recapture every bird repeatedly. These features make this population an outstanding one to study effects of age, because know exact ages, and can repeatedly sample individuals as if they were in the laboratory - using this study system as a "natural laboratory".

Thus, the island population overcomes most of the problems one encounters when studying ageing, and trans-generational effects of age.

Another problem occurs when studying trans-generational effects of age: To disentangle the effects of genetic, behavioural, and epigenetics, one needs to conduct a minimally invasive (and unregulated) experimental procedure called cross-fostering. This means a subset of siblings from one brood will be distributed to other broods. That means, some chicks will be raised by parents that are not their own, while others will remain in their original brood. We can then look at how the chicks grow up, and if they are more similar to their foster parents, we can conclude that they these effects are inherited through the behaviour of the foster parents, while if they are more similar to the genetic parents, it is more likely a genetic effect.

In birds where the female sometimes cheats on her partner, there is one more factor at play: a chick can have a genetic mother and father, and a social mother and father. The social and genetic mother is always one and the same bird, but the social and genetic father do not need to be the same individuals - if she cheated, the social father (the male bird tending to the brood the chick was born in) is not necessarily related to the offspring he raises - so called extra-pair offspring. We can detect such extra- pair offspring by taking a small blood sample and conducting a DNA paternity test. So, if we now cross- foster the extra-pair offspring to foster parents, this chick has three fathers: a genetic father, who only contributed his ejaculate, a social father, who took part in the incubation, and who cares for the social mother, and a foster father, who raises the young.

This allows us to make a more differentiated assessment of which effects are at play and allows us to confidently distinguish epigenetic effects of age from those introduced by parental care. Using cross- fostering and the genetic parentage, we can disentangle the effects of genetics, epigenetics, and environment in the house sparrows for most traits that are not highly variable. However, for this all to work well we need birds with large clutch sizes, to improve statistical power. The bird population on the island has a reasonably large clutch size, but it is a limiting factor. As such, I want to also use a different population of songbirds, of a species with an unusual large clutch size.

In short, I use the island population to better understand longitudinal effects of age, and the effects of parent ages on offspring. These birds need to be repeatedly sampled for their blood - as chicks for their DNA and for parentage analysis, and later repeatedly through life for a longitudinal database of biomarkers of age. I use the other songbird population to better disentangle the effects of genetics, epigenetics, and the environment. These birds only need to be sampled during breeding - chicks for parentage and biomarkers, and parents for parentage.

Typically, what will be done to an animal used in your project?

All birds will be caught in their nests before they fledge. We will measure and weigh the chicks, swab feathers and skin for microbiome samples, and then take a very small blood sample – only the blood sampling is a regulated procedure. When they are old enough, they will receive a passive-integrated transponder (PIT-tag) and rings from the British Trust for Ornithology (BTO). The BTO is a research institute combining professional and citizen

science aimed at using evidence of change in wild bird populations, to inform the public, and policymakers. The BTO is the only bird-ringing license granting institution in Britain and considered to be the strictest in welfare and ethics policies in the world.

The PITs are either embedded in their rings or implanted under the skin (99% of all birds receive this treatment). PIT-tagging is an unregulated procedure, and there is research in my study species that this does not cause lasting harm. The use of PIT-tags reduces the need to recapture birds as we know the birds are alive by detecting their presence when they feed at a feeder equipped with an antenna. The combined use of BTO rings and PIT-tags minimises identification errors, increases the amount of data collected and reduces stress to the animals.

After fledging, we attempt to re-capture birds every year, and on recapture we will register, and measure the bird, take another tissue sample for biomarkers (blood from the brachial vein under the wing), and swab again for microbiome samples. Males may be sampled for ejaculates (an unregulated procedure). The blood sample collected by using a very thin needle to pierce the vein under its wing until a very small drop of blood occurs, which we take up with a microcapillary by capillary action (no suction is involved). Then a cotton ball is gently pressed on the vein, and then wing is being held closed for a short while until the bleeding stops (typically within less than 2 minutes). The ejaculate is collected by gently stroking the bird's genital area, which typically induces ejaculation within 1 minute. The ejaculate is again taken up with a microcapillary. After this the bird is set free again.

Due to the nature of the project, some birds will be subject to repeated sampling, but there is at least 3 months' time between repeated sampling (on average 1.5 years).

All birds will be caught following the guidelines of the BTO, and all handling, ringing will be carried out by an experienced and bird ringer licenced by the BTO.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected adverse effects are some handling stress during capture and handling, and potential discomfort by the needle prick and bleeding. The procedures are known to result in no more than transient discomfort and no lasting harm in wild birds.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild for both, house sparrows and blue tits.

What will happen to animals at the end of this project?

- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We study wild animal behaviour. Natural systems are extremely complex, and while population modelling is a very useful tool, one needs data from the wild to parameterize these models. We know nearly nothing about these types of trans-generational effects, this is a large knowledge gap.

Which non-animal alternatives did you consider for use in this project?

I often use population modelling in my work.

Why were they not suitable?

We have no data available for how to implement or parametrise these models. This is basic research, we do it because the parameters are unknown.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We use state-of-the-art, Bayesian statistics to estimate how many individuals we need. Our study sites are chosen such that the populations are small enough that we can cover all individuals – needed for the parentage analysis and to know their exact ages.

We have estimated the number of animals as the maximal possible number of birds that may be present in the two different study sites. However, it is very likely that it will be much fewer birds than this. The nature of studying a wild population means we cannot for sure say how many birds we will catch.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use our past experience in combination with good practise in our field, and statistical power analyses, to ensure that the size of our population is sufficient to answer our research questions. Unfortunately, the NC3R's Experimental Design Assistant is not sufficient for our use as it does not allow for multiple environmental influences in data collected from the wild, nor does it include the complex genetic analyses we run. Therefore, we use specifically made software to estimate the numbers needed for the genetic analyses (e.g. pedantics package for R). We use state-of-the-art statistical models that allows us to make the best use of what data we have, thereby reducing the need to redo experiments. When working with wild animals one cannot tell beforehand which birds will be caught, and whether they will be suitable for analysis. Therefore, using advanced statistical methods that allow us to use all data drastically reduces the numbers of individuals that need to undergo the procedure.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Using already established long-term populations allows us to use a very large amount of already existing data of birds (ancestry, genotype, age, life history traits) that does not need to be collected again.

Similarly, to collect certain behavioural data we use PIT tags that allow to record presence and absence of birds without the need to recapture them, as such we have drastically reduced the number of recaptures and increased the amount of data. We currently work towards an even more efficient system that includes machine learning algorithms detecting individuals from videos, yet this is still in its infancy.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use house sparrows and blue tits. Both model species are suitable models for testing the proposed hypotheses: their basic biology is relatively well-understood, and our study population offers specific advantages. In particular, my island population is a closed population with no emigration or immigration making it an excellent “natural laboratory” in which I can study trans-generational effects of age.

We also in these species can use our accquired expertise from past studies. For instance, we now use PIT-tags and video analysis to reduce unnesseccary assessment of behaviours in captivity. This reduces the time the birds spend in captivity, and thus reduces stress significantly.

Why can't you use animals that are less sentient?

We are studying animal behaviour, ecology, and evolution in wild vertebrates. There is no less sentient replacement for studying the live and wild animals when we want to know their behaviour, reproduction and genetics, and their response to occurring environmental changes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The proposed procedures will have minimal impact and are central to our aim of understanding behaviour and genetics under the most natural conditions possible. We annually review best practices. We run an annual data workshop during which we review our data and assess whether our activity has welfare costs. From these data workshops I myself have published multiple studies showing that PIT- tagging and cross-fostering are not harmful. Studies by others have shown that the regulated procedure, handling, and ringing are not harmful. However, note that these data are reviewed regularly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We comply with the requirements of environmental regulators, the BTO. The BTO is a research institute combining professional and citizen science aimed at using evidence of change in wild bird populations, to inform the public, and policymakers. The BTO is the only bird-ringing license granting institution in Britain and considered to be the strictest in welfare and ethics policies in the world.

The procedures will be undertaken by staff employed on the project; these will include researchers, graduate students, field assistants and technical staff. Both of my current PhD students hold PILs linked with the old PPL, which encompassed the same procedures.

In addition to the relevant modular training all my staff are and will be trained by me and the BTO in field methods. All my staff that will handle, ring and sample birds will be training with our local BTO. It they do not already have one (which is often the case), they will be put forward for a BTO ringing license.

Over 2600 ringers (including myself) are trained by the BTO to catch and ring birds, a crucial quest for conservation. I and my staff are regularly assessed by BTO trainers to ensure our knowledge, skills, and handling protocol is state-of-the-art. Our BTO catching,

handling, and ringing licenses must be renewed annually, so training and assessment is reviewed annually.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I personally regularly review and revise our methods, read the respective literature, and adapt and improve where possible. I also discuss all these in my research group meetings and expect my staff to also keep up with all relevant and current research. We regularly study the impact of our observations on the wild birds on their behaviour, survival, and fitness, as this is crucial for my research to know.

97. Investigation into the inflammatory mechanisms associated with inflammatory bowel disease and intestinal cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Innate, Inflammatory bowel disease, bowel cancer, inflammation, mucosal

Animal types	Life stages
Mice	juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Inflammatory bowel disease (IBD) and gastrointestinal cancer develop through a complex interplay between host genetics, the gut microbiome and environmental factors. Over the last 15 years we have conducted a number of large genetic studies on patients with IBD, which have resulted in the identification of genes that increase an individuals risks of developing disease. The aim of this research project is to discover how these genes increase an individuals risk of developing IBD and cancer. We will focus our study on characterising alterations in the immune response, microbiome and environment, which have all been shown to be relevant to the development of human disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The cause of IBD and gastrointestinal cancers is still unknown even though we have now identified over 200 genes which increase the risk of developing the disease. We now realise that disease occurs through the complex interactions of genetics, microbiome and environmental factors. In order to determine how an IBD causally related gene influences cellular behaviour, the development of bowel inflammation, the microbiome composition and how the environment impacts on these responses requires the use of transgenic mouse models in combination with human studies and in vitro assay systems. Understanding the complex interplay between these systems will allow us to develop better screening protocols and identify new therapeutic opportunities.

What outputs do you think you will see at the end of this project?

Major outputs from this study

1. We will gain a greater understanding of mucosal immunity

2. We will gain a greater understanding of the processes involved in mucosal inflammation and how this can lead to the development of cancer.

3. We will identify drugs and microbes which can manipulate the immune response in the bowel to either regulate the development of inflammation and/or impact on cancer development.

4. All of the results that are generated will be freely available to the scientific and medical community through presentations and published scientific papers.

Who or what will benefit from these outputs, and how?

In the short term 2-5 years

The study will benefit the scientific and medical community through the generation of novel data on molecules previously unrelated or poorly characterised in the fields of mucosal immunity, inflammatory bowel disease or cancer. Investigators in similar fields could make use of the genetically modified mice, immortalised bone marrow cell lines and mini-gut organoids developed during the study.

In the medium term 4-8 years

The pharmaceutical industry will be able to use the data to help design therapeutics that would directly target the molecules we have identified or the processes which they are involved with. These studies would need to undergo testing and screening, which could make use of the genetically modified mice, immortalised bone marrow cell lines and mini-gut organoids we developed during the study.

In the long term 8-12 years

New therapeutics for the treatment of inflammatory bowel disease and gastrointestinal cancer.

How will you look to maximise the outputs of this work?

We will set up collaborations with other investigators in the field and in associated areas which could benefit from our expertise and cell lines. We will disseminate our findings both positive and negative through manuscript preparation, poster presentations and public seminars.

Species and numbers of animals expected to be used

- Mice: 2300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to study the mechanistic role of molecules identified as associated with human inflammatory bowel disease and gastrointestinal cancer you need to use appropriate animal models. These diseases involve a whole host of different cells from the local gastrointestinal tissue and the circulatory cells. It is currently not possible to study these diseases in a cell based system so we have designed the experiments in mice. Mice are the least sentient, and most understood (in terms of gene expression and gene modification and cell labelling) animals that represent mammalian biology. The models we have incorporated have been shown to be very reliable models of human disease and capable of providing mechanistic insight into how inflammatory bowel disease and colorectal develop in humans. The models use adult mice as these have a fully developed immune system and are able to develop and recover from colitis, which replicates the human condition. This makes it possible to study the induction and recovery processes in these animal models as well as test the effects of potential therapeutic compounds.

Typically, what will be done to an animal used in your project?

Typically, genetically modified mice will be bred in our facility and maintained until needed and then transferred into an experimental protocol. An animal enrolled in our experimental protocol will typically be given an agent that generates inflammation within its lower gastrointestinal tract, via their drinking water or via direct oral administration. Animals in these procedures will typically be involved for 2-3 weeks. The mice may also receive an intra peritoneal or intra venous injection of a cancer promoting or immunomodulatory drug, which will alter the development of the gastrointestinal inflammation and development of the cancer. The duration of the cancer experiments are up to 6 months as the process is relatively slow and is designed to replicate the human condition. Typically a mouse will

undergo 2 (one of which is breeding) and in some instances 3 procedures (Breeding > Bone Marrow Transplantation > Bowel/Sterile Inflammation/colorectal cancer development).

What are the expected impacts and/or adverse effects for the animals during your project?

The mice will develop a colitis like inflammation in their colon which will last for approximately a week in wild type mice and this may differ for genetically altered animals. During the colitis animals will experience moderate discomfort and will lose body weight and show signs of a loss in grooming.

These symptoms will be closely monitored and now allowed to exceed our severity limits. The colitis typically fully resolves after two weeks in wild type mice. In the case of gastrointestinal cancer development, the initial stage involves the injection of a cancer promoting agent in combination with the development of colitis. In the initial two weeks animals will experience moderate discomfort and will lose body weight and show signs of a loss in grooming. These symptoms will be closely monitored and not allowed to exceed our severity limits. The colitis typically fully resolves after two weeks in wild type mice. Over the following 18 weeks tumour will develop in the colon. The tumours will grow slowly and in most cases they will not impede the mouse and normal behaviour, such as feeding and grooming will still continue. They may experience some periods of ill health if they respond poorly to any substance we give them to study/ influence the behaviour of the cancer development. This should be transient, but some mice may experience some weight loss or digestive abnormalities, but this should not persist. Tumours should develop by the 20 weeks, but animals will be sacrificed once they start to show signs that the disease has progressed to an intolerable stage before this time point if necessary.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect that all of the mice involved in the experimental protocols (~75%) will not exceed a moderate severity level and mice used for breeding purposes (~25%) we expect will be mild or sub- threshold levels.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We only perform experimentation on animals when there is no human equivalent or where it would be impractical to study humans. For example, where specific genetic lesions have been produced resulting in the deletion or modification of a protein that might be important in a regulatory signal transduction pathway.

Which non-animal alternatives did you consider for use in this project?

Where possible, we use cells from sources other than laboratory animals. In my laboratory we are doing many experiments in human volunteers and patients with inflammatory bowel disease. These studies have included culturing cells from peripheral blood, rectal biopsies and injection of bacteria subcutaneously. It is our preference, and greatly to our advantage, to use human rather than animal cells. They are cheaper, can be obtained in much greater quantities, and to a higher level of purity. We are also now using CRISPR/Cas9 and RNAi technologies to attempt to deplete cells of specific gene products as an alternative to animal gene targeting studies.

We have also introduced mini-gut organoids that allow us to study the immune mechanisms of the colonic epithelial cells. This approach does require a small number of mice (1-2 per strain), but the mini-guts can be maintained in culture for months greatly reducing the numbers of mice needed for our experiments.

When we acquire a new mouse strain we now generate an immortalised bone marrow cell line which can be used in place of the traditional bone marrow derived immune cells. This means we do not have to breed mice in order to generate a continual supply of these cells during the project.

Why were they not suitable?

It is not possible to substitute the colitis and inflammation derived cancer mouse models for an in vitro (organoid/cell line) alternative. Both models require the complex interplay between the tissue, immune system, circulation and the gut microbiome. We are able to conduct mechanistic studies in vitro and have set up the required systems to do these without the need for large numbers of mice.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Most of the mouse models and protocols are already well established in our project. We have generated a substantial amount of experience at running these types of experiments and have used this knowledge to make sure we collect the maximum amount of information and data from the minimum number of groups and animal. We have identified the most relevant time points during the models of colitis and sterile inflammation induction and this information will allow us to design experiments with the minimal number of animals needed to provide robust and statistically relevant data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In all instances we will adopt the best possible practice on the principles of experimental design whenever possible.

In circumstances where we need to investigate new substances, we will adopt the following best practices:

- The initial doses of any test substances or organisms will be determined through literature search.

- Pilot studies will be performed, starting at a low dose and then increasing doses of substances or organisms for each appropriate route of inoculation, in groups of 2 animals, to establish an effective dose for test substances or appropriate bacterial load (Colony Forming Units – CFU) for each strain of animal. Initially the mutant mice will be infected with a dose of organisms below that producing symptoms in wild type animals and the effects observed in 2 mice. Not all organisms will be tested with all therapeutic agents. In general, the response to all agents will be determined with one organism. Positive effects will be examined with the other organisms.

- Once the dose required to produce a clinically identifiable response has been established, the groups of animals will be increased to numbers which provide sufficient numbers to enable identification of any significant differences.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In close collaboration with our biological services staff we will maintain an efficient breeding programme. All of our mouse strains will be on the same genetic background, which means that wild type control mice can be shared across multiple GAA experiments. Wherever possible, we have attempted to perform multiple measurements from the same dataset (applying the appropriate corrections) and to compare multiple treatments to the same control, reducing the number of control animals required.

We based our sample sizes on estimates of effect size and variability from previous data from our laboratory. However, this is just an initial estimate, and we fully expect that our understanding of the data will increase over the project. As this happens, we will refine our

sample size calculations and either use less animals, if possible without reducing scientific significance, or seek permission to enrol more animals in the study if higher numbers are needed.

We have implemented a new research protocol that can generate an immortalised cell line from a single mouse (Nat Methods. 2006 Apr;3(4):287-93). Each mouse strain has a corresponding cell line generated and these in vitro lines are used for validation and mechanistic studies without the need for additional mice. We will also generate mini-gut organoids from a single mouse from each strain. The mini-guts can be maintained in culture for many months and can be used to study cell-cell interactions and epithelial cell immune responses. The use of mini-guts will reduce the numbers of mice we need to breed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All methods used in these studies use standard protocols which have been published and the overall severity has been determined as “moderate” for all experiments. Methods have been chosen that most closely resemble the human diseases we are interested in. They will provide results which will increase our understanding of human inflammatory disease. If the animals appear ill (hunched, depressed, lethargic, exhibit pilo-erection or become anorexic) for more than 24 hours, or loses 20% of their body weight, they will be killed by Schedule 1 method. Ill animals will be offered particular care. If deemed necessary by the NVS and NACWO, extra bedding material and soft diet may be provided to keep the animals comfortable. The levels of colitis causing drugs and bacteria have been determined through a number of pilot studies previous performed by our group. The levels chosen will produce the minimal pain, suffering and distress on the wild type mice, but importantly still permits us to study the immune response and diseased tissue. The effects in genetically modified animals will be unclear and pilot studies on groups or 2 mice will be used to ascertain the levels needed in these animals to generate the desired response.

Why can’t you use animals that are less sentient?

In order to study the development of inflammatory bowel disease and inflammation driven cancer we require an organism with a similar immune system to humans. Mice are the obvious choice as they can be genetically manipulated and have been shown to develop

colitis and colorectal cancer that closely replicates the diseases in humans. We plan to manipulate the immune system and the majority of drugs and modulators have been developed either in mice or humans. Due to these reasons the use of less sentient animals would not be appropriate in this project.

We have used zebrafish larvae in previous studies to study bacterial infection in the gut, but this model cannot be used in this instance due to a lack of a suitable model for inflammatory bowel disease and colorectal cancer. In addition, a number of genes we are looking at are only expressed in mammals making the fish and invertebrates unsuitable for the study.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all of our protocols we maintain a high level of monitoring during periods when the animals are at a risk of moderate levels of suffering. In addition, all of our procedures have well defined end points, which involve culling the mice with an appropriate schedule one method. This is at a specific time point or at an earlier humane endpoint based on the level of suffering.

In some instances, ill animals will be offered particular care. If deemed necessary by the NVS and NACWO, extra bedding material and soft diet may be provided to keep the animals comfortable. In the event that the diagnosis and any subsequent treatment taken on NVS and NACWO advice failed to remedy the ill-health of the animal, it will be killed by an appropriate schedule one method.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our Biological service unit has a series of guidelines regulating many of the procedures routinely done to animals, for instance how often an animal can receive an injection, where, and how much, or how often it can give a blood sample. We will follow all of these, as well as other standard operating procedures that were developed by specialists at our institute for the explicit purpose of minimising animal suffering, and are periodically updated.

How will you stay informed about advances in the 3Rs, and implement these advanceseffectively, during the project?

Our university routinely circulates advances in the 3Rs and hosts seminars on experimental design and 3Rs. We will always seek to identify ways the 3Rs can be incorporated in our project, while ensuring they do not impact on the (statistical/ biologically relevant) consistency of our data.

98. Cell surface receptor functions in fertility

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

receptors, fertility, reproduction, sperm, egg

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to increase understanding of the role of cell surface and secreted proteins in fertility.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infertility affects 1 in 7 couples in the UK, and also just under half of all pregnancies in the UK are unplanned. These issues cause enormous emotional stress and result from an incomplete understanding of the biological process of fertilization and fertility.

What outputs do you think you will see at the end of this project?

We anticipate that at the end of this project, we will have identified new fertility genes that will contribute to the generation of new scientific knowledge that will be published in scientific journals. This information could also be used to diagnose infertility and thereby provide answers and guide the most appropriate fertility treatments for infertile couples.

Who or what will benefit from these outputs, and how?

Our work on surface molecules in fertility has identified two new genes (Juno and Adgrd1) that can already be used to diagnose infertility in women who are unable to conceive. Within the short term, perhaps within the lifetime of this licence and the next few years, we envisage that further work in this area will increase the number of fertility genes that can be used in these diagnosis. Over the longer term (several years) the research described here will contribute to strategies to develop new fertility treatments and contraceptives.

How will you look to maximise the outputs of this work?

All of the data from our animal experiments will be published in open access journals thereby increasing the exposure and reach of this research to other scientists across the globe. The research is regularly presented to other members of the scientific community in conference presentations and seminars. Where appropriate, upon publication of the research a press release will be made to inform the general public of the advance. We will also use some of the more recent vehicles for publication which enables the publication of negative data to maximise the dissemination of our research.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the research outlined in this project will use mice as an animal model. Mice are a suitable model for these studies because they are mammals, and almost always contain a recognisable counterpart to any human proteins that we identify in our in vitro screens. Mice have a long history of making important contributions to the understanding of human biology and many valuable resources such as gene- deficient mice are available. Therefore they enable us to make scientific advances with an increased confidence of

correctly interpreting the outcomes of experiments designed to discover new treatments and therapeutics to improve human health. We will mainly use adult mice because they are fertile but some work on eggs and embryos will be necessary.

Typically, what will be done to an animal used in your project?

Animals will receive vaccinations with the aim of inducing an immune response to generate antibodies. Typically, individual mice will receive an initial vaccination followed by two booster immunisations. It will usually be necessary to take small amounts of blood to assess the antibody responses. These procedures are very similar to those that are given to babies and children as part of their routine vaccinations. Animals will be superovulated by the injection of hormones similar to the procedure used to superovulate women undergoing fertility treatments. In a few cases, surgery will be used to gain access to the reproductive tissues so that the mechanism of infertility can be established; for example, this will address if there a problem with sperm-egg fusion, embryo transport, or embryo transplantation.

What are the expected impacts and/or adverse effects for the animals during your project?

For the majority of procedures, we expect that the animals will experience no more than mild transient discomfort during the procedures. Where surgery is required, this will be done under general anaesthesia and the latest surgical techniques.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect that any procedure will exceed the moderate category. The vast majority of procedures performed under this licence (>95%) will cause only mild and transient discomfort to the animal, we estimate that the remaining 5% will experience moderate levels of suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to make antibodies because non-animal alternatives typically result in antibodies whose properties are not adequate for our work. We need to use animal models to study fertility because there are no suitable alternative models for sperm and eggs that would allow us to study fertilization nor investigate the transport of embryos in the mammalian oviduct.

Which non-animal alternatives did you consider for use in this project?

We have considered the use of some antibody selection systems that do not require the use of animals, but these alternatives aren't adequate for our research. To study fertilization and embryo transport, there are no suitable alternatives that do not require the use of animals.

Why were they not suitable?

We have found that antibodies generated without animals often have lower binding strengths and so they are not suitable for our purposes (see Appendix 1 for a scientific justification). It is not currently possible to use non-animal alternatives to investigate fertilization or the transport of embryos through the oviduct.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Making good quality antibodies requires a significant investment in research time and we will not generate more than three antibodies per year. Each antibody would usually require immunising around five animals to ensure at least one animal has an appropriate immune response. Allowing for additional attempts, this totals ~50 mice over 5 years.

To investigate fertility, the group sizes used will be established according to effect size of the phenotype under investigation. For those genes that result in complete sterility, which are the genes of greatest interest to us, the group sizes can be kept small because they have such a strong effect size. In these instances, the group sizes are usually less than ten. We estimate that we will use no more than 250 animals for these experiments: 200 for superovulation and 50 for embryo transfers.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals that we require to make antibodies, we have developed a method of making more antibodies at one time in fewer animals. This works by immunising animals with mixtures of proteins and identifying those secreting specific antibodies at the selection stage. To produce oocytes (eggs) for IVF assays, we will use superovulation to increase the number of eggs that we can obtain per mouse, which reduces the total number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where we require multiple monoclonal antibodies at once, we will consider co-immunising mice with protein mixtures to reduce the number of animals that we require. For fertility investigations, we may perform one type of experiment on one of the two oviducts. Where appropriate, we can use the other oviduct from these mice for other types of experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using adult mice to study sperm - egg interactions using in vitro fertilization. It is necessary to obtain sperm and eggs from mice to acheive this. Sperm and eggs are obtained by a terminal procedure and so there is no long-term suffering or pain. Where we need to access the female reproductive tissues using surgery, we will do this using general anaesthesia and the latest surgical techniques to minimise any suffering or pain.

Why can’t you use animals that are less sentient?

We cannot use animals that are less sentient than mice because we require the use of a mammalian species which contains an adaptive immune system to make useful antibodies. For our fertility research, it is known that mice contain orthologous proteins to humans that are involved in fertility and so the discoveries that we make in mice can be applied to humans - this would not be the case using fruit flies or nematodes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be refined by continual improvement and discussions with the scientists and animal technicians, NACWO and NVS to create a culture where the welfare of the animals is central to experimental design. Regular training and protocol review will ensure that any refinements will be implemented promptly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The National Centres for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) web site provides a resource of up-to-date protocols, publications, guidelines, videos and other information that enables the rapid dissemination of the most up-to-date best practices.

Discussions with other scientists at conferences and within the establishment will ensure that improvements in local practices are quickly implemented. Examples of where procedures have been recently refined are the single use of needles, providing enrichment in the cages (fun tubes, mirrors, blocks, food as seeds), adopting tube handling, and use of animal body cues such as the grimace scale to observe pain.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly discuss refinements for animal experiments with the NACWO, NVS, and animal technicians. We will keep abreast of developments discussed and implemented by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) through their monthly newsletters and blogs. The animal facility holds regular forums and updates on the current best practice and latest developments.

99. Cell biological pathways in neurodegeneration

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Late-onset neurodegeneration, Cell biological pathways, Primary cultures, Genetics, Tissue analysis

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will establish cell biological function and dysfunction of biological pathways that are implicated by genetic analysis, including genome-wide association studies (GWAS), in late-onset neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). A particular focus will be on changes in the movement of cellular components along cellular membranous structures and signalling function in neuronal and immune cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

AD and PD are degenerative disorders of the brain that lead to progressive physical decline and cognitive impairment. Currently there are no disease modifying treatments for either condition that stop or slow disease progression. PD and AD affect 4% and 20% of the population over the age of 80 respectively, and so represent an increasing healthcare challenge as the life expectancy of the UK and global population increases. As the disease progresses, brain dysfunction and neuronal death increases, but the reasons why this occurs are not clear. This is an important gap in our knowledge, as in order to develop new treatments that can halt or slow neurodegeneration, we need to understand the molecular events underlying pathogenesis. The ultimate goal of this project is to lay the foundations for the development of novel drugs that can slow down the rate of disease progresses and improve the quality of life for people suffering from late-onset neurodegenerative diseases.

What outputs do you think you will see at the end of this project?

We want to define how neurodegeneration risk factor proteins come together to control cell biological function, and how inherited genetic changes in these proteins causing neurodegenerative diseases such as AD and PD disrupt this. This new information will be made available to scientists, patients and carers at national and international conferences and open access publications.

Who or what will benefit from these outputs, and how?

Our ultimate goal is to use this information to lay the foundations for the development of novel drugs that can slow down the rate at which neurodegenerative diseases progress, delaying the worsening of symptoms and improving the quality of life for people with these diseases.

How will you look to maximise the outputs of this work?

My work will benefit from ongoing long-term national and international collaborations with cell biologists and electrophysiologists on Parkinson's and Alzheimer's disease respectively. In addition, recently strengthened national collaborations through shared supervision of PhD studentships with molecular neurobiologists and bioinformaticians, who have extensive experience in cell biological pathways in late-onset neurodegenerative diseases will support the research strategy of this project further.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project requires the use of juvenile and adult mice to establish the underlying mechanisms and progression of neurodegenerative diseases. Mice are the species of choice primarily because they are routinely used for laboratory investigations of genetic defects in the CNS, for which a considerable amount of experimental data on neurodegeneration has already been gathered. Mice are also the standard species for generating genetically modified animals. The genetically modified mice we use have shown no clinical signs to date. In addition, all chosen procedures cause in general no harm to the animals. In the unlikely event of any adverse effects the mice will be treated. If this fails to alleviate symptoms mice will be humanely culled. The data we generate in mice can provide more information about human disease states and can be compared to other studies using models of neurodegeneration. We will generate tissue cultures and use tissue from these animals for our experiments. This will provide a reliable and consistent source for generating data on the underlying mechanisms and progression of neurodegenerative diseases.

We have collaborations with colleagues working on PD genes in drosophila that inform our work in mice and also considered further the use of invertebrates for our aims. However, use of drosophila and C.elegans while in general useful for investigations of neurodegeneration are not suited for our investigations. For example, whereas mice and humans carry the LRRK2 PD and LRRK1 genes, drosophila and C.elegans only carry one lrk gene. In addition, cell signalling such as Wnt signalling is less complex in invertebrates in comparison to mice/humans. e.g., drosophila only carries one dsh gene, humans/mice possess DVL1, DVL2 and DVL3. The consequence is that loss of function of Wnt components in dropsophila leads to often severe developmental changes whereas phenotypes in mice can be mild e.g., DVL1 knockout mice show mild behaviour changes.

Typically, what will be done to an animal used in your project?

Typically, mice with genetic modifications (GA mice) causing neurodegeneration in patients will be bred. The offspring will be used for the generation of primary cell cultures. Some mice will be injected with lentiviral biosensors to record activation of different cell signalling pathways. The injection of lentiviral biosensors is preferred to backcrossing the animals to diverse cell signalling reporter strains as this would require the use of additional animals for every signalling pathway investigated.

Alternatively, mice will be genotyped to identify wild type and genetically modified mice. These mice will then be aged for different amounts of time and terminally anaesthetised to obtain brain tissue for further analysis. In a subset of experiments we will treat these mice with lipopolysaccharide (LPS) to mimic infection/immune stimulation and thereby

reproducing conditions considered to be important in the environmental genetic interaction leading to neurodegenerative diseases through immune modulation.

What are the expected impacts and/or adverse effects for the animals during your project?

The transgenic mice used in our experiments have not exhibited or are expected to exhibit any abnormal behaviour or show signs of distress. These mice breed and develop normally. Further, terminally anaesthetising mice to obtain the brain tissue is expected to cause minimal distress.

Some mice will have to undergo injections of biosensors to record cell signalling changes and/or LPS to induce peripherally neuroinflammation and potentially increasing neurodegeneration in our genetic models. These are standard procedures causing potentially mild discomfort at the injection side and potentially mild to moderate reactions in response to the LPS induced inflammation.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The breeding of transgenic mice and obtaining brain sections from mice is likely to cause minimal distress and is therefore likely to be at most of mild severity. This will apply to approximately 70% of the animals that we will use.

We estimate that 30% of will undergo LPS and/or biosensor injections. Injections might cause pain categorised as mild as it might result in some temporary discomfort to animals. LPS can potentially cause inflammatory reactions that could be mild or moderate. However, the C57BL mouse strain we use is resistant to this effect of LPS.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

One of my key questions is what happens when cell biological functions, including signalling cascades, are up- or down-regulated in vivo? In order to address this, we will

investigate changes in a complex cellular context e.g., innervation from different brain areas and also interaction of the brain with peripheral and CNS immune cells. In addition, PD and AD are late onset slow progressive disorders. Therefore, pathogenic changes need to be observed over longer periods of time during development and ageing. Currently, complex interactions between different components of the immune system, different cell types in the CNS and communication between peripheral and different CNS regions cannot be achieved in cell cultures. However, we will whenever possible use cell culture methods to inform in vivo treatments.

Which non-animal alternatives did you consider for use in this project?

In vitro experiments will whenever possible be conducted in immortalized cell lines including patient derived induced pluripotent stem cells (iPSCs) if applicable to inform experiments in animal models.

We also use in silico advanced computer models in particular Protein Protein Interaction (PPI) network and expression analysis including data from post-mortem human tissue to inform our animal experiments.

Why were they not suitable?

In-vitro assays cannot adequately model the complete array of molecular, cellular, physiological and behavioural interactions necessary to fully understand how genetic modification results in normal or abnormal processes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have taken precautions to reduce the number of animals to the minimum required for reproducibility and to obtain quantitative data from our analyses. We establish conditions for assays on wild-type mouse tissue to reduce the use of GA animals and provide preliminary data on expected variations.

This way we obtained data on 3-6 animals per group showing significant ($p<0.05$) differences in protein levels between GA and control animals.

Our work using immune modulators requires us to establish optimal conditions before any reliable statistical calculations can be attempted. Nonetheless, published literature suggests that differences between genetically modified animal models of

neurodegeneration and controls will be more pronounced under activated than under basal conditions, suggesting that required numbers will be no more and likely less than the number required to establish significant differences under basal conditions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will be able to use several brain areas, additional organs and/or blood for immune cell isolation form the same animal reducing the total amount of animals required. We estimate that one animal is required to produce three of the required data sets e.g., in brain, spleen and bone marrow derived macrophages.

The minimum number of animals needed to achieve statistically significant results will be based on the statistical analyses. If possible, means and standard deviations from previous experiments or the literature will be taken for power calculations. However, when working with novel immunomodulatory treatments, preliminary experiments (cohort no larger than 3) will be carried out. Animals will receive a specific dose of e.g., LPS and compared with a matched control group. The optimal dose may then be adjusted, and further animals added. This will also provide information on effects and potential side effects of different components. Once repeatability is gained for components with minimum severity the dose will be applied to an experimental cohort. This will reduce total animal number and constantly refines our protocols.

If indicated we will perform power calculations using programmes such as G*Power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Evaluation of outcomes from preliminary experiments will determine subsequent group size as defined by power calculations using standard deviations from the preliminary experiments. This will be done using recognised statistical packages using a statistical significance of at least $p<0.05$ and a power of 80%.

Experimental design will include appropriate controls to allow detection of the effect of change of individual variables.

Statistical analysis will be performed using both parametric and non-parametric methods depending on data distribution. For the use of multiple groups we typically utilise an ANOVA test, for comparisons of 2 groups we often utilise a student’s t-test.

We ensure that any tissues generated from previous experiments are archived and stored appropriately ensuring that unnecessary repetition of experiments is avoided.

As part of good laboratory practice, a protocol for each experiment including statements of the objective(s); description of the experiment, including experimental treatments, the size of the experiment, and experimental material; and an outline of the method of analysis of

the results will be obtained prior to acceptance of experimental protocols by the PPL holder.

Animals will only be bred if a user requirement has been established, and the breeding programme will be subject to regular review to optimally meet anticipated demand. Spare animals will be made available for use on other scientific projects. Breeding will be optimised, wherever possible, to produce only the genotype required e.g. Homozygous breeding pairs.

Most experiments, involve breeding transgenic mice. These are heterozygotes to generate a mixture of wildtypes, genetically modified mice and heterozygotes. We use wildtypes and genetically modified mice for our experiments. However, we cannot determine the numbers of these generated by a given breeding pair. From our experience, we need to keep at least 4 breeding pairs per colony to allow generation of the required numbers of animals for all our experiments and for the colony to be maintained. Hence, the number of mice estimated for breeding is based on our current experience of the number of mice produced using 4 breeding pairs per mouse colony.

Animals used for breeding and experiments are group housed in enriched environments. This allows animals to be relatively relaxed, thereby ensuring that we get good quality tissue from them and obtain the maximum amount of data from each animal.

For pharmacological agents that we have not used previously and for which there is no publicly available data, we will carry out pilot experiments to optimise these experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the most appropriate model species for this investigation as they are the lowest animals in the evolutionary tree with suitable models of PD and AD cell biology and signalling. No use of species phylogenetically closer to humans is proposed.

We will be using genetically modified mouse models for our work. The GA mouse models of familial neurodegenerative diseases we have chosen are well documented, readily available and used as disease models by researchers worldwide. The genetic modifications have been shown to have little impact on breeding and the animals have a normal lifespan and are able to eat and drink normally.

Mice will be genotyped where possible using ear snips. Under exceptional circumstances such as genotyping of neonates where due to limitations of DNA extractions genotyping might not be possible using this method, tail snips might also be used to genotype animals if required. To reduce distress via this method, we will apply a local anaesthetic to the tail end before obtaining the snip

Other than injections the mice will not receive any surgical intervention. For example, lentiviral injection at the first day of life (P0) is a short procedure, and therefore animals recover instantly. We have used lentiviral biosensors over the last 5 years and have not observed any adverse effects.

In general, components anticipated to be used under this PPL will consist of routinely used compounds such as LPS, and compounds modified from routinely used compounds including compounds with neuroprotective potential. Therefore, administration of any compound is not expected to produce more than mild adverse effects.

We will use treatment to modify protein activity in cells. We will only do this if the agents have been shown to be safe and not cause any long-lasting harm to animals. In particular, some transgenic mice will be administered routinely used immunomodulatory substances such as LPS. Any compound previously not in routine use will be tested in a small number of animals prior to being applied to an experimental cohort to refine protocols so that repeatability is gained with minimum severity. To minimise adverse effects of injections, we have optimised procedures where necessary by using appropriate local anaesthesia, aseptic techniques and administering analgesics to reduce infection if required. We find that using these conditions, animals are not distressed.

To quantify the effects of genetic modifications or treatment, we will obtain tissue from animals. To obtain good quality tissue, we will terminally anaesthetise animals before embarking on this procedure. This will ensure that the animals experience little distress during this process.

Why can’t you use animals that are less sentient?

We will be investigating cell biological function when possible by setting up primary cultures from embryonic stages or mice at the first day of live (P0) or the following day (P1). We will be investigating effects of genetic and environmental changes on late onset neurodegeneration. Therefore, we will also require adult mice to obtain tissue after terminal anaesthesia.

We also considered the use of invertebrates. However, use of drosophila and C.elegans while in general useful for investigations of neurodegeneration are not suited for our investigations. In addition, we have collaborations with colleagues working on PD genes in drosophila that inform our work in mice.

For example, whereas mice and humans carry the LRRK2 PD and LRRK1 genes, drosophila and C.elegans only carry one lrk gene. In addition, signalling such as Wnt

signalling is less complex in invertebrates in comparison to mice/humans e.g., drosophila only carries one dsh gene, humans/mice possess DVL1, DVL2 and DVL3. The consequence is that loss of function of Wnt components in dropsophila leads to often severe developmental changes whereas phenotypes in mice can be mild e.g., DVL1 knockout mice show mild behaviour changes.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Biological Services unit staff and/or research staff monitor animals housed in the Biological Service unit daily. If animals are observed to be experiencing distress, we monitor these more regularly. The NACWO and/or NVS might be consulted.

For at least 5 days post injection, animals are regularly monitored twice a day for signs of distress. Moreover, they are weighed daily to ensure that they are feeding and developing normally. They are group housed in environmentally enriched cages.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will following Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines which have been published and are available on the NC3Rs website.

In addition, we will follow the published Animal Welfare and Ethical Review Body (AWERB) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I and researchers working with me will continue to attend seminars run by NC3Rs representatives such as regional managers. In addition, we will check blogs, internal and external webpages, and other current or new resources when they become available. We also discuss NC3Rs at our local BSU user meetings. Further, we are in regular contact with the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS) who will also provide advice on how to implement advances in 3Rs during the project.

100. Exploring the role of the cell surface molecule podoplanin in fibroblast-macrophage cross talk in arthritis

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Arthritis, Inflammation, Podoplanin, Fibroblasts, Macrophages

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to explore the role of a new family of cellular targets expressed on fibroblasts and macrophages that enhance joint inflammation or promote its resolution during arthritis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is now clear that the persistence of inflammation can also occur both as a result of insufficient production of anti-inflammatory mediators as well as the more traditional mechanisms of excess inflammatory mediators These observations have important implications for the way in which we treat chronic inflammatory diseases. Much like a diabetic is given insulin to replace the insulin they can no longer make, we could start giving naturally derived anti-inflammatory mediators to individuals with chronic inflammation to restore and replace the ones that they are no longer able to make enough of. In this way we would be replacing good pathways rather than just blocking bad pathways to restore healthy joints.

Biological therapies (such as anti-TNF) have transformed the lives of patients with Rheumatoid Arthritis (RA). However, their effect is transient, and relief is partial. There remains a pressing need for effective, treatments that really cure as opposed to just treat RA. Our patient partners have explained to us that while current treatments are good at reducing inflammation in their joints, they are not as effective in reducing pain and restoring joint function.

Our preliminary work has identified new molecules on the surface of cells that reside in the joint which regulate joint inflammation and help the resolution of arthritis. However more studies are needed to better understand the cellular and molecular mechanisms involved in these processes. In this way we will be able to evaluate the efficacy and safety of reagents that not only block inflammation but also promote the processes that help to resolve it. This will place us in an ideal position to develop new therapeutic strategies that permanently switch off arthritis and ultimately cure inflammatory arthritis such as Rheumatoid Arthritis.

What outputs do you think you will see at the end of this project?

The work to be carried under this licence will:

Drive a clearer understanding of how arthritis starts and develops by evaluating the role of a new family of cells and molecules by exploring the cellular mechanisms that trigger inflammation or promote its resolution.

Determine new cellular and molecular targets related to fibroblast-macrophage cross talk which will help develop new therapeutic strategies to cure RA.

Provide new scientific findings on the mechanisms that drive the resolution of inflammation in RA that we will disseminate to the scientific community through oral presentations and posters as well as high impact scientific publications.

Who or what will benefit from these outputs, and how?

- Pathways to Impact
- Short-medium term

Scientific developments and innovations leading to our enhanced understanding of the role of immune effector fibroblasts in inflammatory arthritis described in this licence will have an immediate impact on the ongoing projects at the host organisation, and further afield upon dissemination. Moreover, the project aims to improve our understanding of the processes driving pathology in chronic disease, specifically disease pathways mediating the persistence of joint inflammation that we anticipate will underpin the development of the next generation of anti-rheumatic therapies.

Medium-long term

We intend to invite external seminar speakers who have an interest in the role of fibroblasts in chronic inflammatory diseases to our organisation, with the view of fostering further collaborations based on the concepts and ideas incorporated in this proposal. We envisage that these collaborations will occur during and following the completion of this project, and therefore represent a medium to long-term impact of this work. Using these collaborations, we will develop the infrastructure needed to translate these findings into clinical trials and ultimately, clinical practice, therefore fully exploiting the translational potential of this work.

Long-term

Clinical academics, pharmaceutical companies and patients directly will benefit from advances in our understanding of disease pathology and particularly why some individuals with arthritis do not respond to currently available treatments, along with identification of novel targets, which can be taken forward to develop new therapies. We will develop collaborative networks to realise the translational potential of our findings over the subsequent 5-10 years following the completion of this project.

How will you look to maximise the outputs of this work?

Collaborations: This project builds on a strong collaboration between three leading institutions. The members of these research teams, with a strong expertise in RA pathophysiology, will provide all the necessary knowledge, skills and abilities to deliver this project and translate the scientific discoveries into patient benefit.

Dissemination of new findings: We and our collaborators are planning to use different approaches to disseminate our research findings and maximise the uptake of our research by the scientific community and clinicians.

Explanation of project aims and presentation of data at professional meetings and conferences by either oral or poster presentations. This will allow us to raise awareness of our work, exchange knowledge and receive feedback that may be helpful for additional studies.

Publications of high impact papers in a peer-reviewed journals to reach the widest possible audience and be available permanently. Negative results will be also published to avoid

repeating experiments that are inappropriate for the topic or do not support the initial hypothesis.

Public engagement: Throughout the project, we will engage and involve patients and the public in our research who will participate in the dissemination of our research and provide a powerful voice. Our group has established patient research partners group who arrange regular seminars and events attended by patients with RA to enable the dissemination of our findings into a diverse audience.

Species and numbers of animals expected to be used

- Mice: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse models of arthritis recapitulate aspects of RA pathogenesis in humans and have contributed directly to the development of anti-rheumatic therapies. Mice are the lowest mammalian model for human skeletal and immune systems in which we can test the impact of gene manipulation and evaluate the efficacy of therapeutic interventions. We use adult mice as they have fully developed joints.

Typically, what will be done to an animal used in your project?

We will use two main models of arthritis; induced (90% mice) and spontaneous genetic (10 %). Within the induced arthritis the majority of animals (80%) will undergo general anaesthesia (up to 10 minutes), daily handling for scoring and calliper measurements of joints and limbs; administration of an agent or cells to determine cellular function or therapeutic efficacy and will be killed up to week 8 via schedule 1 method or non-schedule 1 for withdrawal of fluids (e.g. blood).

Each experimental model will be monitored daily following intervention and mice will be assessed for any signs of distress with supportive measures provided. Procedures will be undertaken using the most appropriate anaesthetic and analgesia will be given. The mode of substance administration will be chosen to cause the least harm and distress to the mouse. Any new substances or route of administration will be tested in a small pilot study and the mice monitored daily for signs of distress.

Humane endpoints will be strictly adhered to.

What are the expected impacts and/or adverse effects for the animals during your project?

The maximum severity limit in this licence is moderate. Possible adverse effects include irritation from local injection, pain from joint inflammation, weight loss or mobility problems. All forms of arthritis cause joint stiffness and some degree of disability for the duration of the study, but refinements will be always provided prior to the start of arthritis experiments. Pain will be also managed using opiate analgesics with advice from the NVS.

Any animal showing deviation from normal behaviour as judged by; body weight, body condition, general and coat appearance, gait or behaviour will be treated with pain relief and administered food supplements in the form of gel or mash. Before pain and inflammation exceed a moderate severity level, animals will be killed to prevent any on-going pain. All animals will be killed at the end of the protocol with the exception of protocols 1 and 2 (Breeding and maintenance of genetically modified mice).

TNF-DARE transgenic mice, the spontaneous model of arthritis used in this project, will also develop inflammatory bowel disease and may exhibit signs weight loss 10-12 weeks of age. These mice will be monitored and scored for arthritis and systemic inflammation, and we will refine our approach with self- administered analgesic gel (but not NSAID) to prevent animal suffering and with advice from the NVS. They will be humanely killed no later than 20 weeks from the first sign of arthritis.

For induced polyarthritis models, animals will be killed by week 8 after first signs of arthritis via Schedule 1 method or non-schedule 1 for blood withdrawal. Not every joint is affected at the same time so these mice will be monitored and scored for arthritis and systemic inflammation weekly and we will refine our approach with self-administered analgesic gel ((but not NSAID) to prevent animal suffering and with advice from the NVS.

Monoarthritis models which are less debilitating than the polyarthritis models affects only one joint and resolves rapidly within 5-7 days but is not as representative of RA as the polyarthritis models. There are no systemic effects expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severity to approximately 95% of animals Mild severity to approximately 5% of animals.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To study the inflammatory response, three components need to be examined: time, place and cell type. While place (organ) and cell type (leucocyte or fibroblast) can be examined relatively easily in humans, it is difficult and, in some cases, unethical to perform multiple biopsies, and adoptive transfer experiments in humans with arthritis without underpinning preclinical data that support therapeutic utility. Furthermore, as manipulating fibroblast biology has the potential to affect systemic immune function, we will need to determine whether manipulating fibroblasts as a therapeutic target affects the innate and acquired immune response and this requires testing in different mouse models and those that most closely resemble aspects of human disease. This step is essential to ensure that the translation of our findings to humans passes through the appropriate clinical regulatory stages before use in patients with arthritis. Where possible, human samples (tissues and cells) will be used to test the efficacy of test compounds using in vitro histo-culture experiments.

Which non-animal alternatives did you consider for use in this project?

Human primary cells/cell lines (isolated from human synovium biopsies) and peripheral blood mononuclear cells (PBMC) isolated from human blood samples will be used for cell isolation and co- culture (organoid) experiments in the presence and absence of an inflammatory stimulus (TNF-alpha) with or without test agents targeting PDPN and its binding proteins. After co-culture, cells and medias will be collected and analysed for genes and mediators known to stimulate or antagonize inflammation and tissue damage.

We are also planning to set-up and use organoids system; 3D spheroids made from synovial cells that are reconstituted with cells derived from synovial tissue for co-culture experiments. This system will provide a powerful tool to study the synovial cellular crosstalk between fibroblasts and macrophages driven by PDPN to test the effect of agents such as antibodies. We anticipate that this parallel approach will reduce the use of animal models.

In this project, we also aim to establish a correlation between podoplanin (PDPN) expressed on fibroblasts and macrophages and changes in disease activity and severity in patients with RA. To perform this, we are planning to use freshly isolated synovial tissue biopsies and matched blood samples from patients.

Why were they not suitable?

In addition to in-vitro models, this programme of work requires models that mimic acute and chronic arthritis that recapitulate the temporal aspects of human RA pathogenesis, allowing us to produce biologically meaningful results. This work includes pre-clinical

therapeutic (drug and/or cell) efficacy studies required prior to embarking human clinical trials. There are no other in vitro alternatives to this work.

We will also keep reviewing the scientific literature in order to identify any new emerging technologies and models that could be potentially adopted instead of in vivo models (such as the 3D organoids mentioned above) .

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal number calculations are based on data obtained from ours and our collaborators previous experiments using the same mouse models of arthritis. All experiments are designed to ensure that minimal numbers of mice are used to obtain biologically significant results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Statistical calculations based on previous experiments will be used to ensure that minimal numbers of mice will be used in this project.

When new procedures are planned, we will use the NC3R's experimental design tool for good experimental design. Each animal will be assigned to treatment groups randomly and experiments will be performed blind with respect to the treatment and genotype in to minimise unintentional variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In vitro methods using human samples will be used initially in all cases to test the efficacy of new compounds and estimate the magnitude of the expected response.

Where therapeutic interventions or new procedures are planned in-vivo, small-scale pilot studies will be first established in 2-4 mice prior to full experiments to assess variability.

We also plan to use, where possible, the monoarthritis and serum transfer induced models of arthritis as opposed to the collagen induced model: both the serum transfer and monoarthritis models need fewer mice for the following main reasons:

The penetrance of disease is significantly higher.

In Monoarthritis model, only one joint is affected, allowing us to use the contralateral joint as a nonarthritic, genetically and environmentally matched control.

In the serum transfer induced model, all four limb joints are affected, allowing us to use one mouse for many readouts

We will stop our experiments at any stage where our findings fail to show any significant increase in our understanding of the mechanism by which the cells and molecules of interest function in inflammatory arthritis. In addition, the literature will be also continually reviewed to ensure that we are not repeating published work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- Mice are the best model for the study of persistent disease because:
- The main components of their immune system are shared by humans; this is essential where immune responses as opposed to the function of individual genes is being studied and thus will produce satisfactory results.
- A wide range of wild type and genetically manipulated strains of defined genetic makeup are available.
- An extensive range of reagents are available for analysis of immune responses.
- They are the most acceptable animal models that show the least degree of neurophysiological sensitivity and will suffer the least pain, suffering, distress or lasting harm.
- There are no other alternatives to this work.

We have refined and streamlined as much as possible the models of arthritis that we use. Importantly we will use a research strategy that includes the use of different models of inflammatory arthritis to recapitulate a specific phase or aspect of joint disease more accurately, depending on the scientific questions of that particular experiment. This targeted approach will limit the use of persistent models of joint inflammation to only those experiments where it is needed to model that specific aspect of the disease.

Where necessary, males carrying the mutation will be used for breeding with wild type females as this means the female does not have the burden of the effects of the mutation alongside pregnancy.

The maximum time a single mouse is likely to have arthritis is 5 weeks as the arthritis is self-limiting. We will give mice treatments that lessen pain and discomfort that might occur as part of the inflammatory response. This will include pre-emptive treatment with opiate analgesia. We have also adapted treatments used in patients with arthritis (e.g anti TNF biologic therapies) for use in mouse strains that have a genetic predisposition to develop arthritis. We have put in place mitigations as described below in the 3Rs to ensure that any suffering by an individual animal is minimized.

Why can't you use animals that are less sentient?

Less sentient animals do not recapitulate aspects of the RA pathogenesis in humans. Adult mice, to be used in this project, are the lowest mammalian model for human skeletal and immune systems in which we can test the impact of gene manipulation and evaluate the efficacy of therapeutic interventions.

We will not be able to use animals that have been terminally anaesthetised as the duration of experimentation (days) exceeds the time that animals can stay under general anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use well established reagents and protocols that have been developed under previous animal licences and in collaboration with other research centres in the UK. We will therefore be able to use the lowest and pre-optimised doses of agents that are well tolerated. Our experience with murine models of arthritis over the last decade as outlined within this project has allowed us to refine them as much as possible by minimising any adverse effects and very clearly defining humane endpoints. In doing so we have developed specific score sheets for the different polyarthritis and monoarthritis protocols in use in order to capture the specific aspects of each arthritis phenotype and ensure clear and consistent analgesia and humane endpoints. Similarly, due to prior optimisation, all mouse models will run for the shortest possible duration to minimise suffering. We have also made refinements to the housing of the animals to cater for any disability arising from arthritis. These refinements include long drinking spouts on water bottles, soft and warm flooring, non-tangling nesting material, food on the cage floor, use of the most refined route of administration, and hydrating food. All these refinement methods will be constantly reviewed to ensure best practice is followed.

The use of analgesia for these protocols will be kept under review and depend of model used and scoring sheets. For example, anti-TNF therapy will be used during the breeding of animals for the TNFΔARE model to reduce their symptoms however in some

circumstances specific analgesic drugs would not be applicable such as in monoarthritis, where the main scientific endpoint is weight-bearing so this will be affected by analgesics.

We have refined a few of our protocols over the last decade years.

● We will use established reagents and protocols that we have developed and refined over the last 5 years to treat the mice. Therefore, we will not need to perform unnecessary toxicity studies and will be able to use the lowest doses of agents that are well tolerated.

● We have refined and streamlined as much as possible the models of arthritis that we use. Importantly we have very clearly defined humane endpoints. We have refined procedures of cellular transfer, including by intra-articular injection, so the smallest volumes can be injected.

● Where necessary, males carrying the mutation will be used for breeding with wild type females as this means the female does not have the burden of the effects of the mutation alongside pregnancy.

Where necessary, male mice, transgenic for the gene of interest will be mated with wild type females in order to exclude indirect effects on the progeny derived from gene overexpression in the pregnant female.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All staff involved in this project will continuously review the literature on animal welfare provided by the local AWERB and be guided by NACWOs and NVS to ensure that all our experiments are conducted in the most refined way.

To ensure the best use of the most appropriate animal models, our experiments will be carried out according to the LASA guidelines: Laboratory Animal Science Association (https://www.lasa.co.uk/) providing guidance toward animal experimentation and welfare.

When planning for animal experiments, we will check the PREPARE: Planning Research and Experimental Procedures on Animals: Recommendations for Excellence (https://norecopa.no/PREPARE) checklist to ensure an optimal study design and be aware of different elements to consider before and during the study.

Finally, we will follow the ARRIVE guidelines: Animal Research: Reporting of In Vivo Experiments (https://arriveguidelines.org/) for reporting and describing our in-vivo experiments when writing scientific publications to ensure a comprehensive and clear description useful for other research projects using animal models.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will attend local 3Rs events and sign-up to the NC3Rs e-newsletter to stay informed about the latest 3Rs advances and updates.

101. Genetic analysis of metastatic cancer

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, metastasis, therapy, genetics, imaging

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main objective of this project is to reproduce genetic alterations observed in human cancer in a mouse system in order to better understand the basic biology of cancer and the process through which the cancer spreads from the initial site to various other organs. Specifically, we will utilise a highly refined set of genetically engineered mice to test the importance of specific pathways in driving disease progression in cancer. Ultimately, success in this work will represent a significant advancement in the field of cancer research through the analysis of tumours and new therapies in a realistic environment.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

More than 1 in 3 people in the UK will develop some form of cancer during their lifetime. Fortunately, the overall survival of most cancer patients increased remarkably in the past decades through early detection, but also improved treatment protocols that include the

surgical removal of tumour tissue and conventional chemotherapy and radiotherapy. However, the majority of these approaches are associated with severe side effects. Moreover, the prognosis for patients with relapsed and/or aggressive tumours remains extremely poor. Notably, the vast majority (i.e. about 90%) of metastatic cancers that have spread to secondary sites are refractory to treatment and are therefore incurable.

Thus, there is a pressing clinical need for further research, like ours, to identify the basic mechanisms that drive cancer progression. This knowledge is essential to assist the design of future anti-cancer agents and improve the outcome of patients exhibiting aggressive clinical behaviour.

What outputs do you think you will see at the end of this project?

We anticipate that our research will advance scientific knowledge about the formation and progression of tumours. This will be achieved through observational studies of tumour development in murine models that satisfactorily reproduce the human disease. This information will be of significant interest to other scientists in academia and in industry, as the results could open up new possible avenues for the development of anticancer drugs for patients with malignant disease. To ensure the dissemination of our work, we will publish our results in scientific journals and present our findings at seminars and conferences.

Who or what will benefit from these outputs, and how?

The primary potential benefit of this proposed work relates to new knowledge about tumour growth and malignancy. This information will have a major impact in the biomedical research as the data can serve as a basis for the development of rational therapeutic strategies to combat metastatic cancer. Thus, the potential secondary benefit of this work will go beyond basic cancer research, possibly leading to the future development of novel pharmacological interventions.

How will you look to maximise the outputs of this work?

A number of avenues will be taken to explore the medical application of our results within the lifetime of this project. Specifically, we will interact with research business managers employed by the establishment to facilitate interactions with industry and translate the potential value of this project to benefit society as a whole. In addition, we will share best practice with the local and national research community using similar models. In particular, the use of imaging will allow us to define more humane refinement of end points and study parameters and we can share these protocols with other teams working in the same area to enable them to do the same.

Species and numbers of animals expected to be used

- Mice: 19500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice constitute important models for the characterisation of complex cellular processes. One advantage relies on the ability to dissect biochemical reactions in vivo by the use of targeted genetic deletions.

This type of molecular manipulations is particularly successful in mice and has contributed substantially to an increased understanding of biological processes involved in normal development and pathogenesis. In addition, the genetic conservation with humans and comparable disease aetiology make mice highly suited for cancer research studies.

Typically, what will be done to an animal used in your project?

This project will create, breed and maintain mice with appropriate genetic changes. For the majority of animals, we anticipate that these genetic alterations will have negligible adverse effects and be of mild severity. Some mice will be utilised for tumour studies. Substances which are intended to alter tumour growth may be given by a number of routes, e.g. in the diet or drinking water, by direct application onto the skin or by experienced licensees via oral gavage or by needle injection, on one or more occasions. We may also make use of special imaging techniques to view the primary tumour as well as their spread in the living animals. Careful monitoring of tumour bearing mice is crucial for our studies and we have strict criteria when animals are to be killed. In particular, tumours will only be allowed to grow to a particular size and if the animal's normal behaviour is altered they will be killed. At the end of the experiment the mice will be killed painlessly according to an appropriate procedure. Autopsies may be carried out to expose adverse effects undetected by external examination and the information used to refine future studies.

What are the expected impacts and/or adverse effects for the animals during your project?

We will do everything possible to minimise adverse effects relating to tumour growth. In particular, we will not allow mice to carry on with fully developed tumours, but we will kill them in a humane way before any signs of suffering will develop. For this reason, the most likely level of severity in most cases will be mild. However, in some cases, some animals will be moderate for their welfare impact. Any suffering will be minimised by careful monitoring to allow for the early signs detection of general lack of wellbeing. Moreover, if there are indications of ill health from gavage or skin ulceration after substances are

applied directly to the skin, then the animals will be removed from the study and humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In the majority (about 70% of mice) the severity will be mild, but never more than moderate.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to utilise mice because an important component of tumour growth is based on the interactions of tumour cells with the host organism (so called tumour microenvironment).

Which non-animal alternatives did you consider for use in this project?

We will continue to employ non-animal alternatives to complement our in vivo studies in mice. For example, we will confirm the therapeutic applicability of our discoveries using cell culture systems. Moreover, we will test the clinical significance of our results by using credible computational modelling built using publicly available data from cancer patient materials.

Why were they not suitable?

While cell cultures and computer modelling constitute alternative approaches, conclusions drawn using such systems need to be tested in murine models that can simulate the cellular complexity of tumours. Additionally, metastasis (the process of cancer spreading to other organs) happens in vivo in the whole organism and there are no experimental alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of mice utilised in this project will be kept to a minimum by using optimum crossing designs. The demand will be assessed before breeding and crossing. Colonies will only be maintained while there is an experimental plan and funding allocated. Moreover, the group sizes and the replications will be kept to the minimum possible to achieve statistically robust data. Importantly, we will keep consulting with statisticians as new studies begin to ensure that the optimal number of animals is used to obtain meaningful results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Every opportunity will be taken to decrease the number of animals used for each experiment whilst still maintaining the statistical relevance of the subsequent data. In particular, experiments will be designed with repetitive measurements of the tumour volumes in the same animal. This has greater statistical power and animals need only be killed at the end of experiments rather than at each time-point, thereby drastically reducing the numbers of animals being used. The use of non-invasive imaging techniques that allow tumours to be studied in the same animal will also improve our study design and reduce number of animals required to answer specific questions. Moreover, we will always aim at maximising the amount of data we get from each mouse. We may also employ national on-line design resources,

e.g. the Experimental Design Assistant (EDA) tool created by the NC3R, and consult with the NC3R team who provide periodic onsite help, to improve our approach.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

An effective means of achieving reduction will be to avoid producing unwanted mice by carefully regulating our breeding colonies. Breeding will be optimised by replacing breeders before their reproductive performance declines. Wherever possible, colonies will be organised to produce only the genotype required by our studies. Cryopreservation will avoid wastage from the need to maintain colonies by continuous breeding. We are very familiar with the tumour models included in this application. Together with considerable experience and expertise available in animal husbandry, this will ensure careful management of colonies, matching supply to demand and thus avoiding the production of surplus animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is one of the best model systems in cancer research that has greatly contributed to advance knowledge and improve treatment. This is largely due to the availability of different models that exhibit distinct cancer phenotypes consistent with the human disease. This advantage will allow us to utilise the most appropriate and refined systems for discovering clinically relevant mechanisms that drive or suppress the development of tumours.

Why can't you use animals that are less sentient?

An invertebrate model would not be appropriate since cancers do not arise in invertebrates and other differences in metabolism would greatly reduce the significance of findings for humans. The mouse is the lowest vertebrate in the evolutionary tree that can be genetically manipulated to produce the required genetically modified model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We operate within a very tightly regulated, clean and well administered facility that has an excellent track record for animal care and safety. In all cases, we will avoid pain, suffering or lasting harm to the animals by the use appropriate anaesthetics and procedures.

Frequent monitoring will be used to assess progression of tumour burden, animal health and behaviour, so humane end-points are reached well before onset of clinical adverse effects. Moreover, we will be kept inform of any potential harmful events through our online system, so that we are always aware of issues with our animals and can respond to them quickly. Overall, adverse effects associated with tumour studies will always be limited to the minimum required for a valid scientific outcome.

Administration of compounds will be carried out by appropriately trained and skilled personal licence holders who will use specifically designed gavage tubes, fine needles and aseptic techniques to minimised stress due to restraint and momentary discomfort from oral gavage or needle insertion. Re- gavaged and repeated injection will be permitted only if the animal has fully recovered from previous procedures. Importantly, cancer therapy will

be given at dose levels known to be tolerated. Any evidence of unanticipated toxicity would indicate reduction in dose and/or frequency of dosing.

Additionally, we will refine our approach by incorporating non-invasive tumour imaging where possible. This will provide opportunities to shorten our experiments, prevent potentially malignant tumours from escaping detection, and ensure that humane endpoints are adhered to, as more accurate detection and analysis of tumour growth informs decision making. Animals will have full recovery between periods of anaesthesia, rehydration and maintenance of body temperature during imaging sessions.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be guided and directed by the most up to date documents on the welfare and use of animals in cancer research. Additionally, we will follow relevant ARRIVE guidelines to ensure that our studies are reported in enough detail to add to the knowledge base.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Main sources of information include:

i) The NC3R team who provides very helpful advice;

ii) The NC3Rs and the BSF newsletters sent by email to all users of the animal facility;

iii) The National Centre for the Replacement Refinement and Reduction of Animals in Research (https://www.nc3rs.org.uk/resource-hubs); and

iv) Peer-review scientific papers, oral communications/posters in conferences and discussion amongst scientific colleagues, reporting alternative in vitro models to the use of live animals.

102. Host-pathogen interactions and vaccines

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

receptors, vaccines, pathogens, host-pathogen interactions

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand the role of pathogen cell surface and secreted proteins in host interactions and develop vacccines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The interactions between receptor molecules displayed at the surface of cells are critical in the regulation of infection biology. This research is focussed on understanding the biological roles of these receptor molecules in infection and identifying vaccine targets.

What outputs do you think you will see at the end of this project?

The outputs of this project will be the functional characterisation of novel extracellular host-pathogen interactions during infection and the identification of new vaccine candidates for clinically and economically important parasites including Trypanosoma spp., Leishmania spp. and Schistosoma mansoni.

Who or what will benefit from these outputs, and how?

The short-term beneficiaries will be other scientific researchers who will gain a wider knowledge of the biology of the pathogen. In the longer term, small holders living in Africa who depend on livestock animals for their milk, food and draught power would benefit from effective vaccines to protect their livestock animals. Ultimately, vaccines for animal African trypanosomiasis would stimulate the economic growth of many African economies. For the parasitic diseases that affect humans, the development of an effective vaccine would have an enormous humanitarian benefit to those living in endemic regions. The timescales to licensure of human vaccines, however, can be as high as 20 years.

How will you look to maximise the outputs of this work?

All of the data from our animal experiments will be published in open access journals thereby increasing the exposure and reach of this research to other scientists across the globe. The research is regularly presented to other members of the scientific community in conference presentations and seminars. Where appropriate, upon publication of the research a press release will be made to inform the general public of the advance. We will also use some of the more recent vehicles for publication which enables the publication of negative data to maximise the dissemination of our research.

Species and numbers of animals expected to be used

- Mice: 3300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All the research outlined in this project will use mice as an animal model. Mice are a suitable model for these studies because they are mammals, and almost always contain a recognisable counterpart to any human proteins that we identify in our in vitro screens. Consistent with this, many pathogens that infect humans also infect mice with similar

pathogenic outcomes (e.g., Schistosoma mansoni, the parasitic worm that causes schistosomiasis in humans). Thus, mice can be used as an appropriate model for preclinical work. Mice have a long history of making important contributions to the understanding of human biology and many valuable resources (such as gene-deficient mice) are available. Therefore, they enable us to make scientific advances with an increased confidence of correctly interpreting the outcomes of experiments designed to discover new treatments and therapeutics to improve human health. We will use adult mice because they have a well-developed immune system.

Typically, what will be done to an animal used in your project?

Objective 1: Animals will receive vaccinations with the aim of inducing an immune response. Typically, individual mice will receive an initial vaccination followed by two booster immunisations. It will usually be necessary to take small amounts of blood to assess the antibody responses. These procedures are very similar to those that are given to babies and children as part of their routine vaccinations.

Objective 2: Animals will be vaccinated as described above and then challenged with a pathogen to determine if the test vaccine candidate works. To quantify how much pathogen is present, we can use an advanced form of imaging where a chemical is given to the infected animals and reacts with a gene in the pathogen to emit light. The administration of the chemical is done by injection and will be repeated several times during the course of the infection.

What are the expected impacts and/or adverse effects for the animals during your project?

For most (>95%) procedures, we expect that the animals will experience no more than mild transient discomfort during the procedures. In some cases (<5%) we may observe complications which could include irritation at the site of injection, and stress symptoms from pathogen infections that unexpectedly exceed established thresholds; in the latter case, this is estimated to last for no longer than a few hours.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

We do not expect that any procedure will exceed the moderate category. The vast majority of procedures performed under this licence (>95%) will cause only mild and transient discomfort to the animal.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Objective 1: We need to use animals to make antibodies where in vitro alternatives have not been found to result in antibodies suitable for our work, or to select mouse monoclonal antibodies for our passive transfer studies where there are no suitable in vitro alternatives.

Objective 2: We need to use animals in our preclinical vaccine screening approaches because there are few or no adequate in vitro infection models available for the pathogens that we wish to study. A vaccine candidate that showed efficacy in an animal model would also lend significant weight to its candidature for progression towards a human vaccine.

Which non-animal alternatives did you consider for use in this project?

For objective 1, we considered alternatives such as phage, yeast and mammalian display. For objective 2, one considered option was to add antibodies to pathogens directly rather than directly infect the mice.

Why were they not suitable?

For objective 1, we have found that antibodies produced using in vitro methods are not suitable for our purposes because they usually have low binding strengths. We must use antibodies that have long binding half-lives (hours) to withstand the stringent wash steps of the staining protocols. A scientific justification is provided in Appendix 1 but is due to the limit on transformation efficiency of E.coli (which limits library sizes to just ~109 clones) and there is no well developed method of affinity maturation which can replace the in vivo process of somatic hypermutation. Where the antibodies are required for passive transfer protection experiments in our mouse infection models to determine the immunological mechanism of protection then we will request dispensation to use mice for monoclonal antibody generation. This is becasue it is essential that we use mouse monoclonal antibodies to avoid any host immune responses to the delivered antibody which would confound our analysis. We are not aware of any available in vitro selection systems that use mouse antibody backbones since almost all are humanised antibodies.

For objective 2, adding antibodies to parasites directly does not avoid the use of animals since they are needed to raise the antisera. Using these models also restricts the vaccine mechanism of action to antibodies and would not appropriately model other aspects of the immune system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

For objective 1, we will not generate more than two antibodies per year. Each antibody would usually require immunising around five animals to ensure at least one animal has an appropriate immune response. Allowing for additional attempts, this totals ~50 mice. We will also need to generate polyclonal antisera in mice for use in passive transfer experiments which is estimated at 50 mice.

For objective 2, we envisage that we will be testing up to 20 vaccine candidates per year. We estimate that we will use 2000, 1000, and 200 animals for the Trypanosome, Leishmania and Schistosoma vaccine screens respectively.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For objective 1, to reduce the number of animals that we require to make antibodies, we have designed and developed a method of making more antibodies at one time in fewer animals.

For objective 2, we will use the very latest technologies to accurately quantify the number of parasites in individual animals over time without killing any animals consequently leading to a reduction in the number of animals used. Where a new host-parasite combination is being used, we will use pilot experiments to establish the inter-individual variability of the infection and attempt to reduce this so that smaller group sizes can be used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For objective 1, where we require multiple antibodies at once, we will consider co-immunising mice with protein mixtures to reduce the number of animals that we require.

For objective 2, we will ensure that pilot studies are performed to refine infection protocols to reduce the inter-individual variability between animals within a cohort. This typically can include selecting the most appropriate route of infection, for example using intravenous rather than intraperitoneal delivery, or direct inoculation rather than by insect bite. In some cases, we are asked for pathogen samples from other researchers and we will use tissue samples taken from animals from control groups which is an example of tissue and sample sharing.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have refined the protocols for the percutaneous infection of mice with Schistosoma mansoni by developing an anaesthesia apparatus that has reduced the stress to the animals during these infection protocols. We have also adopted, where possible, the use of light-based imaging to accurately and objectively quantify parasitaemia - this has worked particularly well for Trypanosoma spp. and Leishmania spp. infections. This has the major benefit that the efficacy of a vaccine candidate can be assessed without the infection proceeding to the stage that adverse effects are experienced.

Why can't you use animals that are less sentient?

We require the use of a mammalian species which contains an adaptive immune system that has clear similarities with the immune system used in the target species for our vaccines that include humans and livestock animals. We must use adult animals that have a fully developed and functioning immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will be refined by continual improvement and discussions with the scientists and animal technicians, NACWO and NVS to create a culture where the welfare of the animals is central to experimental design. Regular training and protocol review will ensure that any refinements will be implemented promptly.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The National Centres for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) web site provides a resource of up-to-date protocols, publications, guidelines, videos and other information that enables the rapid dissemination of the most up-to-date best practices.

Discussions with other scientists at conferences and within the establishment will ensure that improvements in local practices are quickly implemented. Examples of where procedures have been recently refined are the single use of needles, using lances (thin needles) for blood biopsies rather than tail snipping, providing enrichment in the cages (fun tubes, mirrors, blocks, food as seeds), adopting tube handling, and use of animal body cues such as the grimace scale to observe pain.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly discuss refinements for animal experiments with the NACWO, NVS, and animal technicians. We will keep abreast of developments discussed and implemented by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) through their monthly newsletters and blogs. The animal facility holds regular forums and updates on the current best practice and latest developments.

103. Investigating the mechanisms driving cardiac fibrosis and its effects on cardiac function

Project duration

4 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cardiac fibrosis, heart remodelling, fibroblast growth factor 23, arrhythmia

Animal types	Life stages
Mice	adult, pregnant, neonate, embryo, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to characterise the pathways by which fibrous connective tissue in the heart, termed cardiac fibrosis, leads to cardiac dysfunction and to identify novel therapeutic interventions to prevent or reverse cardiac fibrosis.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Due to ageing populations and lifestyle changes, the total number of deaths caused by cardiovascular diseases (CVDs) is increasing – by ~21% from 2007-2017. High mortality rates as well as high level of morbidity contribute to the high economic burden of CVDs.

The treatment for CVD is increasing over time, with prescriptions and operations costs around £6.8 billion in England, the majority spent on secondary care. Centre for Disease Control (CDC) data in US show that Americans suffer 1.5 million heart attacks and strokes each year, which contributes more than $320 billion in annual healthcare costs and lost productivity. By 2030, this cost is projected to rise to $818 billion, while lost productivity costs to $275 billion.

Many of these deadly and debilitating CVDs are associated with cardiac fibrosis. Cardiac fibrosis contributes to both electrical and structural remodelling of the heart which ultimately leads to decreased cardiac function, heart failure and arrhythmias. However, with no efficient treatments directly targeting cardiac fibrosis, it presents an ever-growing clinical challenge.

Our work and that of others has identified a protein called fibroblast growth factor 23 (FGF23) to be associated with the development of cardiac dysfunction in patients with CVDs, particularly in patients with heart rhythm dysfunction (atrial fibrillation) and reduced kidney function (chronic kidney disease). Our in vitro data suggest that FGF23 can promote accumulation of fibrous connective tissue in the heart. This is likely to contributes to cardiac electrical dysfunction and lead to development of debilitating and lethal heart disease. We propose that interrogation of this new mediator of cardiac electrical dysfunction will illuminate novel cardiac biology insights and therapies targeting cardiac fibrosis and thereby reduce patient suffering and death.

What outputs do you think you will see at the end of this project?

We will publish the data in relevant academic journals and present at national and international conferences. The studies may demonstrate utility of the therapeutic interventions for treatment of cardiac fibrosis and electrical dysfunction. If they do, we will look to patenting the products.

Who or what will benefit from these outputs, and how?

Short to medium term we expect that the knowledge gained from this project will be shared with the scientific and clinical community and published in peer reviewed journals (output expected during the project). Thus the academic community will be the primary benefactor of this work. Once the mechanisms of cardiac fibrosis development have been studied and been better understood, the results will be used to inform the clinical colleagues of these findings and work with them on determining whether similar mechanisms also drive cardiac fibrosis in patients (output expected after the project).

The long term benefit of the work will be a better understanding of the mechanisms by which cardiac fibrosis develops and whether it can be treated using the therapies targeted against the FGF23. This will open new avenues to reduce cardiovascular disease risk for example in atrial fibrillation or chronic kidney disease (output expected some 1-5 years after the project), with the ultimate aim to prevent or ameliorate high cardiovascular morbidity and mortality.

How will you look to maximise the outputs of this work?

Publication in the relevant scientific literature and presentation of the data at international meetings is likely to be the primary output for this work. Existing collaboration with our clinical colleagues will also be further strengthened. The intention is that this work generates valuable datasets that will inform clinical practice and may provide a novel therapeutic avenue for the treatment of cardiac dysfunction in patients suffering with atrial fibrillation and with chronic kidney disease.

Species and numbers of animals expected to be used

- Mice: 2300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the lowest mammalian species with sufficiently similar cardiovascular physiology to that of humans, allowing transfer of findings to man. We have however introduced a human mutation that allows for improved sensitivity of mice to commonly used therapeutics, mimicking the response seen in humans. Specifically, a human mutation has been introduced in the gene responsible for sodium handling. This mutation will allow us to better translate our findings.

Adult mice of a specific age range (8-20 weeks of age) will be chosen to minimise variability.

Typically, what will be done to an animal used in your project?

Some animals (less than 20%) will undergo minor surgery to implant an osmotic mini pump or MedRod implant under the skin to chronically deliver recombinant FGF23 or vehicle control for up to 6 weeks.

Alternatively, an animal may be given two doses of FGF23 (or vehicle control) daily for seven days via subcutaneous/intraperitoneal injection. These animals will then undergo non-invasive assessment of structural and functional changes to the heart. This will include echocardiographic imaging and ECG monitoring under anaesthesia before recovery. Blood sampling will also be carried out to measure any circulating factors influenced by FGF23. In the majority (if not all of the animals), the heart will be excised under terminal anaesthesia for further Langendorff perfusion or cell isolation for assessment of cardiac and cellular function to maximize the data obtained from each animal.

What are the expected impacts and/or adverse effects for the animals during your project?

We will use CD-1 wild type mice or a genetically modified mouse line. The mouse line we plan to use are usually not expected to experience any adverse effects. However, if any of the mice develop unexpected phenotypic changes, the animals will be humanely killed.

Based on published data, the dose of FGF23 planned under this PPL has been shown to successfully cause activation of downstream signalling pathways, leading cardiac hypertrophy, with no other reported side effects in the mice.

To test the effects of a therapeutic intervention, some mice will be treated with FGF23 alone, a FGF23- targeted therapy, FGF23 and a FGF23-targeted therapy, or vehicle control (phosphate buffered saline). Pharmacological agents may be administered either by mini-pump/MedRodTM implantation or intraperitoneal/subcutaneous injection.

The agents administered will not exceed the recommended maximum volumes for dosing. FGF23 safety profile when injected subcutaneously, intraperitonealy or intravennously in animals is well documented. However, if animals display signs of toxicity such as reduced mobility and grooming or up to 20% weight loss compared to the controls, the terminal experiment will be performed or mice will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Most animals (80%) will undergo a non-recovery procedure, as they will only undergo heart extraction under terminal anaesthesia.

Small number of animals (20%) will experience moderate severity, due to cumulative effects of FGF23 delivery, echocardiography and heart extraction under terminal anaesthesia. However, every effort will be made to minimise any adverse effects.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cardiac electrical function is complex, involving the interaction of multiple factors, including many cell types and physical and neurohormonal local controls, that cannot currently be studied without animal models. Our understanding of the processes involved, and their relative importance, limits our ability to use computer modelling, though this is a goal we are working towards.Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially be adopted in order to replace in vivo animal use.

Which non-animal alternatives did you consider for use in this project?

We have explored the use of stem cell-derived cardiomyocytes. Work on stem cell-derived cardiomyocytes continues in my lab and we are currently replacing animal work where possible. For example we have just successfully differentiated human induced pluripotent stem cells into cardiac fibroblasts and are starting to use these as alternatives to mouse primary adult cardiac fibroblasts.

Why were they not suitable?

Stem cell-derived cardiomyocytes, either in 2D or 3D culture, do not fully recapitulate the complexity of the heart, including many cell types and physical and neurohormonal local controls.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Expected effect size will be determined through consultation of the literature, previous experience of techniques proposed or through small pilot experiments when possible.

We have used statistical methods to calculate how many animals we need to get meaningful data. This was determined based on the number of different FGF23 concentrations we plan to investigate in vitro, on differential effects on atrial and ventricular chamber of the hearts.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

When designing the experiments, we performed statistical analysis to ensure we use the minimum number of mice per group that will be informative. Importantly, we also employ a variety of approaches to reduce animal usage, including:

1. Using littermate pairs to reduce variability of genetic and behavioural background

2. Performing and analysing experiments in a blind fashion to reduce investigator-induced bias

3. Using standardised experimental methods to reduce variability

4. Optimising tissue usage through the use of innovative technology, such as RNAseq, optical mapping, novel cell isolation technique, etc..

5. The NC3Rs EDA tool will be used to design experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To maximise the information gained from a single animal we aim to perform multiple in vivo and in vitro analyses. Where possible, cell line work and in vitro manipulations have been designed to yield the maximum possible information and reduce animal use.

Better reporting of research should result in better science and more effective use of animals in experiments. Therefore, our findings will be reported (using the ARRIVE guidelines) in the scientific literature and at conferences, thereby minimising risk for future unnecessary animal experiments conducted by others.

Furthermore, literature will be continually reviewed to ensure that we are not repeating published work and that our hypotheses are based on the most up to date knowledge.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Majority of animals will be used for non-recovery work. Where this is not the case, our experimental protocols have been developed to limit harm to the animals, being as short as reasonably possible. We will continue to make efforts to refine protocols and further reduce the welfare costs. Current best practices (e.g. needle sizes used will be kept to a minimum, use of minipumps to deliver FGF23) will always be followed.

The use of osmotic mini pumps to deliver the protein of interest over a long period of time (up to 6 weeks) reduces the need for repeated handling and injections and therefore reduces the distress caused to the animal. Analgesics will also be given pre- and post-operatively to ensure the least pain is felt by the animal following surgery. Strict aseptic procedures will be used in order to prevent infections.

Echocardiography will be carried out under general anaesthesia (up to 30 minutes) so as to limit animal distress and equally to produce better quality data . As the animal is likely to only undergo this procedure up to 2 times over the course of the 6 weeks of treatment it is more stressful to the animal to try and acclimatize it to the imaging process without anaesthesia.

The apparatus for ECG/Blood pressure measurements is already refined to ensure the least distress to the animal i.e. tunnel used to secure animal in natural position and kept in dark throughout procedure.

Blood sampling will follow published guidance on suitable volumes which can be taken while minimising harms to animals.

In addition before conducting each experiment, it will be discussed with the NACWO and NVS to ensure animal welfare is maintained throughout the experiment and that minimum suffering is caused to acquire the scientific endpoints. We will also review each experiment on completion to see what lessons can be learned from the study in terms of endpoints (scientific and humane) and any animal welfare issues that may have arisen during the experiment that could then guide the next experiment.

Why can’t you use animals that are less sentient?

The mouse is the least sentient mammal that is most similar to humans in terns the way their heart works.

Majority of our work will use animals that are terminally anaesthetised. However, experiments studying the long terms effects on the heart cannot be done in terminally anaesthetised animals. For some experiments we will also use cellular models of disease however, these cannot completely replace the need for the use of mice as they cannot replicate the complexity of the whole animal physiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Majority of animals will be used for non-recovery work so will only ever experience minimal suffering. This is possible due to our state-of-the-art optical mapping technologies, allowing for monitoring of heart electrical activity ex vivo.

To minimise the welfare cost to the animals, we will use good animal husbandry, and will provide enriched environment in the cage to keep the animals happier. We will also better handling techniques to reduce the distress that animals experience during handling.

For all procedures, the mice will be carefully monitored and if adverse events are observed, monitoring will increase in frequency and steps, steps will be taken to alleviate them or the affected mice will be humanely killed.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the PREPARE guidelines to ensure our experiments are planned and conducted in the most refined way. LASA guidelines will be consulted to ensure correct dose volumes are selected for the specific route of injection/blood sampling. We will also stay up to date with the NC3R website resources and guidance on the best and most refined practices. All the data will be published according to the ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regularly monitor the guidance given by the NC3R's website, making use of the online resources to ensure the project is carried out efficiently and using the best methods for animal welfare.

104. Treatment of chronic inflammatory disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

rheumatoid arthritis, therapy, inflammation, immunoregulation, autoimmunity

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop new treatments for immune-mediated inflammatory diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is estimated that up to 1% of the population worldwide suffer from rheumatoid arthritis, with around 7% suffering from some sort of autoimmune disease. Furthermore, these figures are rising due to increased longevity and an ageing population. This project seeks to develop new ways to treat these conditions and to improve the quality of life of patients suffering from immune-driven inflammation.

What outputs do you think you will see at the end of this project?

Although there have been considerable improvements in the treatment of chronic inflammatory diseases like rheumatoid arthritis, there remains considerable unmet need. Many patients do not respond adequately to current medications and even in those who do respond, disease usually relapses when treatment is withdrawn therefore there is a need for continuous therapy. The principle objective of this research is to develop new treatments that provide long-term reduction in disease activity without the need for continuous drug administration. During the course of the project we aim to test new therapies which could progress through to human clinical trials. Hence the primary output will be new compounds for subsequent evaluation in man. We predict that new drugs will be identified that are effective in reducing pain and helping patients to lead more active and productive lives. In addition to the development of new therapeutic approaches, this research will provide information about why inflammation fails to resolve in some individuals, leading to chronic disease. This information will add to existing medical knowledge, resulting in scientific publications and presentations at international meetings.

Who or what will benefit from these outputs, and how?

It is envisaged that this research will provide the scientific rationale for carrying out clinical trials of at least three new compounds or forms of therapy for patients with rheumatological diseases within a period of five years. If these trials are successful, the wider patient population should benefit within ten years. This benefit would be in the form of improved treatments, aimed towards disease remission, and improved quality of life. In the shorter term (within five years) the scientific community will benefit through disseminated knowledge, leading to a greater understanding of chronic inflammation.

Companies may also benefit through the identification of therapeutic targets, enabling new drug development opportunities. In the longer term, the scientific knowledge gained from this project will also benefit patients with other autoimmune diseases, including multiple sclerosis and type 1 diabetes, as many pathological processes are shared by multiple inflammatory diseases.

How will you look to maximise the outputs of this work?

We aim to collaborate fully with clinicians to determine priorities and maximise outputs. Collaboration with other research groups is also key to making progress and will form a major part of our work. In addition, we aim to collaborate with the pharmaceutical industry to expedite the translation of basic research findings into new drugs for patients. We will attend national and international meetings and disseminate and discuss our work in an open and honest manner. Peer-reviewed papers remain the major mechanism for disseminating our research findings and we also aim to share our findings with patients via patient participation groups.

Species and numbers of animals expected to be used

- Mice: 13000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Inbred strains of mice will be used in this study. Mice are suitable because they are known to develop autoimmune arthritis in a similar manner to humans. Furthermore, there are a wide range of reagents available for mice which means that disease processes can be studied using conventional laboratory practices. Mice also have a relatively short life-span and it is possible to study factors present during development which could predispose to disease in adulthood.

Typically, what will be done to an animal used in your project?

Mice will be bred and maintained. A proportion of the mice will be immunised with an antigen in order to provoke an autoimmune response. These mice will develop arthritis which will be treated with novel anti-arthritic drugs. Blood samples may be taken and non-invasive imaging may be performed under anaesthesia. The mice will be humanely killed at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Arthritis causes pain and limping but is short-term. Mice with arthritis are normally maintained for 7-10 days and then killed. Analgesics will be administered during the arthritic phase where possible. Some of the drugs may have unexpected adverse effects and these will be monitored carefully.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For the mice on breeding and maintenance protocols we expect the majority to experience subthreshold severity (approximately 85%), and a small number to experience mild severity (approximately 10%) and less than 5% to suffer moderate severity. For the mice on experimental protocols, we expected the majority (approximately 80%) to experience moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animal models are necessary because arthritis is not a static process confined to a single tissue and culture techniques do not offer a realistic alternative. Rather, the inflammatory process is dynamic and highly complex, involving trafficking of cells from distant sites to the joint via the circulation. Hence, modelling the effects of treatment on arthritis must at some stage involve whole animals, rather than isolated tissue extracts.

Which non-animal alternatives did you consider for use in this project?

Where possible we use cells and tissues from patients and we have considered a synovial cell culture systems to study the effects of anti-arthritic therapies.

Why were they not suitable?

Synovial cell cultures do not model the whole body. They are useful for studying the effect of a treatment on a particular cell type but they do not inform us about the effect of the treatment on arthritis.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Statistical methods are used to predict the number of mice per group needed to demonstrate a significant effect.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The use of animals will be minimised through careful planning of experiments and by the use of cell based culture systems whenever possible. The use of NC3R's Experimental Design Assistant is used to plan experiments and all experimental plans are discussed in

detail before embarking on the work. Mouse colonies will be kept at a minimum to ensure that the minimum number of mice is used throughout the study. Animals will only be bred to meet the experimental requirements and both sexes are likely to be used for experiments.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The maximum amount of data possible is gleaned from each experiment through the use of next generation sequencing and other technological advances. This means that we don't have to repeat experiments to get more information. We also perform pilot studies with very small numbers prior to embarking on big experiments. This ensures that ineffective compounds are weeded-out at an early stage. In addition, our breeding strategy ensures that we breed the minimum number of mice possible to achieve the goals of the study.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Human rheumatoid arthritis may last for decades, resulting in years of reduced quality of life. In contrast, we will use mouse models of arthritis that are of short duration (7-10 days) and therefore have limited impact on lifetime suffering. Furthermore, wherever possible we will use the most refined arthritis model, that affects only one joint, and we will administer analgesics to alleviate pain. Where possible, genetically-altered strains will be used with which have no harmful phenotype.

Why can't you use animals that are less sentient?

Mice are the least sentient species for which animal models of arthritis have been developed. Fish are unsuitable as they lack synovial joints.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We aim to improve pain management through increased use of analgesics, provided that they don't influence scientific endpoints. Specifically, a blocked trial design will be devised

to establish whether metamizole and buprenorphine can be used to alleviate pain without affecting arthritis. If both approaches are unsuccessful, we will trial the use of pregabalin, gabapentin and amitryptyline as analgesics. Another important refinement is to minimise the amount of time that animals are maintained with arthritis. Hence acute, rather than chronic, models will be used wherever possible. Mice with arthritis are provided with moist palatable food at cage floor level to ensure that their nutritional requirements are met. Mouse colonies will be kept at a minimum to ensure that the minimum number of mice is used throughout the study. We aim to perform further research into the milder adjuvants than complete Freund's adjuvant, which will be used as little as possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will also consult the NC3Rs website on a frequent and regular basis and will follow the guidance laid out in the following article, of which I am a co-author.

Hawkins P, Armstrong R, Boden T, Garside P, Knight K, Lilley E, Seed M, Wilkinson M, Williams RO. Applying refinement to the use of mice and rats in rheumatoid arthritis research. Inflammopharmacology (2015) 23:131-50.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Regular meetings are held within the University to keep abreast of developments in the 3Rs. I am a reviewer for the journal: ALTEX: Alternatives to Animal Experimentation, and receive regular notifications of significant articles from them. Regular contact is maintained with the Named Information Officer. Within our research group, we discuss animal experiments on a regular basis to ensure that best practice is maintained.

Home Office

105. Determination of cns efficacy and safety pharmacology

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Central Nervous System, Efficacy, Safety, Pharmacology

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this Project is two-fold. Firstly, the safety evaluation of test substances which may induce adverse side effects on the central nervous system. Test substances may be novel chemical entities (NCE's) as potential new drugs.

Secondly, to determine the efficacy of therapeutic substances interacting with the central nervous system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Disorders of the Central Nervous System (CNS)/Mental illness is a growing problem, with 1 in 4 people experiencing mental health issues each year, with an associated economic and social burden.

● Depression is a leading cause of disability worldwide, often occurring in conjunction with other mental health issues and associated with suicide and heart disease.

● Anxiety affects a significant number of people, and over recent years has been seen to be increasing among young people.

● Psychosis and schizophrenia are also on the increase, with the latter being increasingly connected to cannabis consumption at an early age.

Such conditions can be hugely debilitating for the individuals and challenging for care givers and family. Although a number of treatments are available for such conditions they are often associated with varying degrees of side-effects. For example, anti-psychotics may cause weight gain, suicidal feelings/behaviour, seizures, sedation, neuromuscular effects, emotional effects and heart problems.

As such, new medications are required which have improved or equal efficacy with lower associated side-effects.

For the treatment of Schizophrenia, medications have primarily targeted the positive symptoms (e.g. hallucination, delusions, disorganization speech and behaviour), however, more recently, there has been an increase in drugs being developed to treat the negative symptoms of this condition (e.g. lack of emotion, low energy, low motivation and impairment of social interactions).

Data from this project will be used to improve human health by supporting the development of new medicines for these and other related disorders, and also assessing novel drugs for their potential to produce unwanted behavioural/CNS side-effects and support the requirements of regulatory submissions to ensure drugs are safe for humans to take.

What outputs do you think you will see at the end of this project?

The benefits of this programme will be two fold. Firstly it may contribute to the development of new drugs that help alleviate CNS disorders that are common in society, like anxiety, depression and epilepsy. These new drugs may work better in the clinic, relieve or cure diseases and have better side effect profiles. We may, by our work, also

contribute to better knowledge and understanding of these types of disease, and that knowledge may be used to develop further new drugs.

Secondly, the models on this project may be used to assess the safety of a test material, and find a dose that causes no effect, or identify an unwanted effect. This is important when planning future trials in humans, to make sure any starting dose in a clinical trial is safe for the patients taking it.

Who or what will benefit from these outputs, and how?

In both the medium and long term, patients will benefit by having newer, modern and safe drugs that are more effective than the current options.

How will you look to maximise the outputs of this work?

The work will be shared with customers who will use it to determine their future strategy, or for submission in documents required by regulatory authorities. Whilst we have no direct control over what happens to the data after we have shared it, we trust from information given to us that it is used for regulatory purposes or to support regulatory purposes (e.g. to support drugs progressing to clinical trials). Previously however, we have collaborated with customers and shared data we have produced in the form of Scientific publications that are in the public domain.

Species and numbers of animals expected to be used

- Mice: 4500
- Rats: 7000
- Guinea pigs: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most of our experiments will be carried out on mice and rats as these are the smallest relevant species that we can use that have an central nervous system (brain and nervous system) that is comparable to humans. In some specialist cases we may use other animals (the guinea pig) because what we are trying to find out is better done in that particular species rather than in the rat or mouse.

The only other time we would use a species other than a mouse or rat is to continue work that has been previously done in that species. For instance if previous work, and results gained, had been carried out in a guinea pig, it would make no scientific sense to start the next stage of a programme of work in a rat or a mouse.

We will be using adult animals in our studies, as we do not expect to be investigating CNS drugs for use in young animals or children.

We very occasionally use guinea pig pups for a specific test to measure anxiety. This is a well characterised model, dependent on the pups being separated from their mothers for short periods, hence we have to use animals of this age (the test won't work with adult animals).

Typically, what will be done to an animal used in your project?

Typically on this project, animals are dosed over a short period of time (sometimes only once, rarely longer than a week) with test materials, and observed and tested for effects on their nervous system before being humanely killed. These observations include measurement of activity and alertness, and a lot of them don't actually require us to even touch the animal as they are observational assessments, or require only minor manipulations. Most studies would last a matter of days (much less than a month) although some, very occasionally may last for longer than that.

Dosing of animals is commonly done orally using a flexible tube, or by injection using a syringe and needle, maybe directly into a vein, or into a muscle into the arm or leg, or just under the skin.

Blood samples are usually taken from easily accessible veins in the neck or the tail of rats or mice. We are limited to how much blood we can take at once or over a month. If we need a large blood sample, we would do this when the animal is anaesthetised and we would not let them recover consciousness.

Where possible, we try and take any of the tissues and samples we need after the animals have been humanely killed.

Sometimes we need to induce a condition in animals to test if a new drug can alleviate the condition. For example if we are looking at a drug to treat people having convulsions, we have to give an animal a drug to make them convulse, after we have given the test drug. We use a drug we have a lot of knowledge and experience of, so we know the right dose to use, and constantly watch an animal when it is being administered it via infusion. As soon as an animal starts to convulse, we stop dosing and kill the animal immediately, to stop any suffering. We sometimes also need to assess whether or not drugs affect the 'sleeping time' of an animal when dosed with a known sleep inducing drug. This is a test to ensure drugs don't depress brain function on their own. When animals are sent into sleep we watch them, and keep them on a warm surface until they have recovered, when they are humanely killed.

In one protocol we also have to surgically prepare animals by cannulating a vein so we can administer test materials directly into the animals bloodstream. These surgeries are usually very rare, and are carried out under the supervision of a vet, by a trained surgeon. We take as much care to avoid any infection in our animals as a surgeon would if

operating on a human. We 'scrub up' and wear mask, gowns and gloves just like they would. We watch our animals when they are recovering from surgery and give them extra heat and bedding and food and water until they have recovered. Animals would also be give supportive medicines like pain relief as needed, on the direction of a vet.

We very occasionally use guinea pig pups for a specific test to measure anxiety. This is a well characterised model, dependent on the pups being separated from their mothers for short periods, hence we have to use animals of this age. No overt distress is caused to the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

When dosing an animal by injection or taking blood, The amount of pain an animal feels is similar to what a patient would feel having an injection done by a doctor. If we have to repeatedly inject animals using a needle and syringe, we would choose different sites to do this where possible. If we can take blood samples when an animal is deeply unconscious then we do. If we need to take repeated blood samples or need to dose repeatedly then we try and use different sites. Of course everyone who performs these procedures are trained to a high standard.

Animals undergoing surgery receive the same sort of care as a patient would in hospital. We discuss their pain relief and use of antibiotics with a vet before we start. We administer drugs as necessary and give them plenty of time to recover from surgery before we use them in experiments.

Sometimes we have to confine animals in tubes for dosing and sampling or special chambers to measure respiration. We can introduce the animals to these situations slowly, and watch to see if they are ok. Usually most animals are absolutely fine in these new surroundings and don't suffer any distress or anxiety.

Any animals who are tested to see if a drug prevents convulsions, are made to convulse with another drug (after we have dosed with the drug with think prevents convulsions). We constantly watch them when giving this drug, and as soon as we see them start to convulse they are killed humanely.

Measuring whether a drug affects 'sleeping time' by administering an agent that causes sleep, can cause animals to get cold because they are not moving around. However we warm all these animals whilst asleep, and as soon as they have met the test we have for them being fully awake, then the experiment is over and they are humanely killed.

Dosing with drugs may cause adverse effects in some studies, although this is rare, as we usually have a good idea as to what doses are safe from other studies. We do observe our animals at least twice a day, and the people who do this know the signs when an animal is ill. If an animal is ill, we would check it more frequently, and get more senior staff involved

in its care for advice, including vets. We also help sick animals out by giving more bedding, more heat and special food to make them more comfortable.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

On the last project, about 95% of animals were classified as having displayed mild severity, due to the procedures they had performed.

It's impossible to predict the proportion of severities expected on a service licence, as this will be dependent on what study types we are asked to perform.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The Central Nervous System is an extremely complex, poorly understood system which controls a wide variety of functions essential to life including breathing and heartbeat. There is no adequate model to replace the whole animal experimental model, as the complex, co-dependent mechanisms under investigation cannot be adequately modelled in vitro.

In many cases the protocols listed in this Project will be used later in the life cycle of a test substance and in many cases, particularly for pharmaceuticals, tests in test tubes will have been conducted previously (often by the Sponsors) to ensure the drugs do what they are meant to, and are safe, before we expose them to testing in animals.

As a service provider we do not own the test materials under evaluation, therefore, using test tube methods for testing drugs is not appropriate. However, available literature is reviewed prior to commencing any animal procedures if required. Prior to conducting animals studies, clients are requested to provide information relating to their test material candidate, together with details of other work performed, relevant regulatory requirements and a justification for conducting in vivo investigations.

For some protocols the licence, the tests are legally required by regulators around the World to make sure new drugs don't have ill effects on the nervous system, otherwise they cannot enter trials in humans.

Which non-animal alternatives did you consider for use in this project?

Although there are presently no non-animal models for behavioural responses, in vitro information e.g receptor binding/functional assays and chemical structural analysis will typically form part of the over profiling of candidate drugs

Why were they not suitable?

Non-animal studies are unable to evaluate behavioural responses to new drugs produced by the nervous system.

To fully assess the pharmacodynamic effects (effects of a drug on the body) of a new drug testing in animals is necessary. Only in a fully operational circulating system can the drug's distribution, metabolism, excretion which may alter or intensify the efficacy or adverse effects of the new medicine be fully understood.

For these reasons animal models remain essential in the development and safety assessment of new medicines.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers we have used are based on figures of previous usage from previous projects, or a projection thereof (based on estimated incidence) based on requests received from customers in the past. It is, however, impossible to accurately predict the number of studies that may be performed, in the circumstances.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All experiments will be designed in order to achieve the scientific objectives using the minimum numbers of animals. For study types that are less well established and for which historical data may not be available, the literature will normally be consulted to help establish the group size. Alternatively, there may be other data to aid this process. The Department of Statistics are often consulted to assist in this process particularly where the study type is not routine.

Whenever possible, common control groups will be used in order to minimise the numbers of groups used.

For less established study types, preliminary pilot studies may be conducted whereby smaller numbers of animals may be used to generate data in order to ensure that the experiment operates to expectations and to generate some data which may be used to optimise the study design.

Experience has shown that occasionally, Sponsors have a preference with regard to their design and numbers of animals to be used. Rationale for the design will be requested from the Sponsor and such designs (particularly where they are at variance with EU requirement or studies usually conducted here) will be discussed internally (and with the Home Office as appropriate) and forwarded to the Department of Statistics for advice. Such advice will be taken into account when determining the design/numbers to be used in the study with the goal of using the least number of animals to achieve the scientific objective.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For studies where a new drug is being tested in animals for the first time, we would often test that in a small group of animals (usually 3-5) to give us confidence that the dose levels we chose are safe, and the drug affects the system its designed to, without making an animal ill. These are called pilot studies.

We will try and get as many outputs as we can from a single animal where possible, without adversely affecting its welfare. So if we need to get a blood sample to confirm the presence of a drug or biomarker, for example, we will often do that in the same animal, rather than use separate ones, when possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Greater than 80% of animals used on a previous project involved a single dose of test material followed by observations which largely involve not actually touching the animal after dosing (a few behavioural tests do involve handling and a mild application of a stimulus). Similarly measuring locomotor activity usually involves placing an animal in a cage using an automated system, or a shorter visual observation and counting of movements by eye.

In some studies we may need to take serial blood samples from animals to find out how the test material is handled in the body. Taking a blood sample from an animal causes about the same amount of pain as having a blood sample taken by a nurse. All of the people who do this work are trained to do this without causing any undue pain or distress.

To test for anticonvulsant activity of a drug, you have to induce a convulsion using a specific drug at a fixed dose. As soon as a convulsion is observed, the animal is humanely killed. During the administration of the drug the animal is watched throughout. Similarly to assess sleeping time (a test to measure if a test material has the ability to stimulate or depress CNS function) then you have to dose the animal with a drug that causes them to fall asleep sleep. We monitor the animals throughout this time, keep them warm, and as soon as the animal wakes up, and we have measured this time, the animals are humanely killed. Again these animals are constantly watched during their sleep and kept on a heated surface to maintain body heat.

In the rare cases we need to surgically prepare animals, we consult a vet prior to surgery to agree doses of pain killing drugs and antibiotics if required. We then perform the surgery using the same principles of sterility and post operative care as if it was a patient undergoing surgery in a hospital.

We very occasionally use guinea pig pups for a specific test to measure anxiety. This is a well characterised model, dependent on the pups being separated from their mothers for short periods, hence we have to use animals of this age. Separating the pups from their mother makes them anxious and allows us to test potential drugs for anxiety, to see if their behaviour changes with or without the drug.

Why can’t you use animals that are less sentient?

Rodents (rats, mice and guinea-pigs) will be used in all of the studies conducted under this licence. Rodents are considered to be of the lowest neurophysiological sensitivity that will allow us to achieve the study aims and are considered suitable for the predicting what's likely to happen in humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animal welfare is of utmost importance and Good Surgical Practice will be observed for any animal undergoing surgical procedures. Surgery will be conducted using aseptic techniques (to prevent infection) which meet at least the standards set out in the Home Office Minimum Standards for Aseptic Surgery. Before we start surgery, we agree with a Vet what pain killers or antibiotics the animals need both before and after the surgery. When recovering from surgery, we give the animals extra heat and monitor them closely until they start behaving normally again. We then check them at least twice daily before they go on study.

In addition, care is taken to provide as much environmental enrichment as possible . This is things like plastic shelters in their cages, blocks to gnaw on, extra bedding for warmth and if they need it, food supplements after surgery.

Dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm and will be the minimum consistent with the scientific objectives of each study. In addition, suffering will be further minimised by implementing clearly defined humane endpoints to prevent excess suffering.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For any surgical interventions, then the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (2017) will be followed.

For blood sampling and dosing then the following guidelines/literature will be followed:

First report of the BVA/FRAME/RSPCA/UFAW joint working group on refinement, Laboratory Animals, 27, 1-22 (1993).

A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes, Journal of Applied Toxicology, 21, 15-23 (2001).

Regulatory guidelines

ICH 7A SAFETY PHARMACOLOGY STUDIES FOR HUMAN PHARMACEUTICALS.

ICH Topic M 3 (R2) . Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

This will be achieved by regular discussions with our Named Information Officer, colleagues in Animals Technology, and by attending appropriate training courses and conferences, or getting feedback from such events.

106. Therapeutic potential of tregs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

T regulatory cells, Therapy, Transplantation, Autoimmunity

Animal types	Life stages
Mice	adult, neonate, juvenile, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to explore novel strategies for increasing T regulatory cell numbers. T regulatory cells (or "Tregs" for short) are immune cells that play a key role in keeping the immune system under control.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to undertake this work as there are many instances where increasing Treg numbers would be beneficial for patients.

For example, increasing Tregs may help prevent the rejection of transplanted organs. Organ rejection occurs when the recipient's immune system "sees" the donor organ as

foreign, triggering a response that ultimately destroys the transplant. Currently patients receiving organ transplants are treated with immunosuppressive drugs which reduce the risk of rejection by dampening down their overall immune response. However these drugs are not fully effective (despite these drugs a significant proportion of patients still reject their transplant), and they have side-effects, including leaving patients more susceptible to infections. Increasing Treg numbers may: (i) reduce the risk of rejection further and/or (ii) allow the dose/duration of immunosuppressant treatment to be reduced, so reducing side effects.

Increasing Treg numbers may also be helpful in treating patients with autoimmune diseases - such as multiple sclerosis. Autoimmune diseases occur when a patient's own immune system mistakenly "sees" their own tissues as foreign, damaging them. In multiple sclerosis it is the coating of the nerves in the brain and spinal cord that is attacked and damaged leading to disability; this coating is called myelin.
Dampening down an over-reactive immune system by boosting the number of Tregs may be a safe therapy to try.

What outputs do you think you will see at the end of this project?

We expect to see several outputs at the end of this project. Including:

1. Generating new information. In particular we will determine if we can increase Treg numbers within our animals using two strategies (a) by transplanting laboratory expanded (outside the body) Tregs into our animals; (b) by giving a drug called IL-2.

2. Publications and presentations. We expect to publish the results of these studies in peer-reviewed scientific journals. We also expect to present the findings of our work at local, national and international scientific meetings.

3. Intellectual property that may support a planned patent application. We have recently identified a novel type of Treg, present in the blood and tissues of humans. In addition to testing "standard" Tregs in our animal models, we will also test these "new Tregs" in this project. We hope this will generate additional data to support our planned patent application.

4. Data to support future funding applications/clinical trials. We are very excited about the potential therapeutic applications of our work. And we hope that data generated in this project will help us apply for additional funding to take this work forward and "nearer to the clinic".

Who or what will benefit from these outputs, and how?

Beneficiaries of this work will include:

1. In the short-term - our work will benefit the clinical and scientific community working on Tregs, including individuals working on strategies to increase Tregs within the body.

2. In the medium to longer-term - if we demonstrate that IL-2 increases Treg numbers in one of our animal models (which will mimic what happens to the immune system of patients with multiple sclerosis (MS) receiving treatment with an immune-depleting drug called alemtuzumab) we will carry out a clinical trial of IL-2 in MS patients treated with alemtuzumab. Alemtuzumab is a highly effective treatment of MS, but it has side-effects.

In particular, 50% of patients treated with alemtuzumab develop thyroid autoimmunity - that is, after treatment, their immune system starts to attack and damage their thyroid gland (instead of damaging the protective coating of the nerves). We believe we can reduce the risk of this happening by boosting Treg numbers with IL-2. If we are correct our work will significantly benefit individuals with MS, allowing them to receive treatment with a highly effective drug, without the risk of complications.

3. In the long-term our work on laboratory-expanded Tregs and our "new type of Treg" has the potential to help patients undergoing organ transplantation, and also patients who suffer from autoimmune conditions such as MS.

How will you look to maximise the outputs of this work?

We will maximise the outputs of our work by:

1. Collaborating with other groups - including groups working on therapies for MS, and groups working on understanding Tregs. We have a strong collaboration record.

2. Disseminating new knowledge - through presenting our work at local, national and international meetings and by publishing our work in peer reviewed journals. We will also aim to publish any novel protocols we develop, and negative data/unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 2,250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

1) We will use adult mice that lack an immune system of their own (such as NOD-scid IL2Rgammanull - also known as NSGs) to answer objective 1 "Are expanded human CD8 Tregs suppressive in vivo?" Because these mice lack an immune system, they can be "humanised". That is - when human immune cells are transplanted into the mice (by injection), they can survive and form a working immune system. This process is called engraftment. We have extensive experience of working with NSGs and know that they are the best type of mice to use for these sorts of experiments.

2) To answer objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?" we will use adult human CD52 "transgenic" mice (hCD52tg) or wild type mice. hCD52tgs have been genetically engineered to express a human version of a molecule called CD52 on their immune cells. This allows us to mimic what happens to the immune system when patients with multiple sclerosis are treated with a drug called alemtuzumab. Alemtuzumab binds to, and depletes, cells expressing CD52 (immune depletion). After treating the mice with alemtuzumab we will see if we can increase Treg numbers by giving the mice IL-2. hCD52tg mice have previously been shown to closely mirror immune depletion and immune recovery seen in humans following treatment with alemtuzumab, so are the appropriate animals to use to address this question. In this project we will also

study the effect of low- dose IL-2 in promoting Tregs following other immune-depleting drugs, including cladribine another treatment licensed for multiple sclerosis. Wild-type animals or hCD52tgs can be used in these experiments.

Typically, what will be done to an animal used in your project?

To answer objective 1 "Are expanded human CD8 Tregs suppressive in vivo?" adult NSGs will receive a human skin graft. During this procedure a small piece of human skin (obtained with appropriate consent, typically from deceased organ donors or from patients undergoing cosmetic surgery) will be stitched onto the mouse's back after removing a very small piece of mouse skin. This will be done under a general anaesthetic and the mice will be given painkillers afterwards if needed. Once the skin graft has healed, the mice will be "humanised" by transferring human immune cells plus or minus Tregs intravenously (i.e into a vein) or intraperitoneally (i.e. into the body cavity). Prior to immune transfer the mice may be exposed to low dose irradiation, that makes 'space' for the incoming human immune cells to grow. Read out from this type of experiment is rejection of the skin graft (from time of humanisation). Transferring Tregs into the mice should delay or prevent graft rejection. Mice may be imaged to see where the Tregs have redistributed within the mice, if done this will be performed under a general anaesthetic.

For objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?" adult hCD52tg mice (and non transgenic litter mate/ wild-type controls) will be treated with alemtuzumab (or other immune-depleting drugs such as cladribine). Following immune depletion, the animals will be treated with low dose IL-2 or placebo (i.e. an inactive sterile liquid) given subcutaneously (i.e under the skin). The ability of low dose IL-2 to increase Treg numbers will be assessed by serial blood tests.

What are the expected impacts and/or adverse effects for the animals during your project?

1) For objective 1 "Are expanded human CD8 Tregs suppressive in vivo?"

All mice will undergo a brief surgical procedure under a general anaesthetic to receive a skin graft. Similar to humans, it is common for mice to be a little "groggy" for a short while post- anaesthesia, and for a day or so they may go off their food slightly. The mice are expected to recover well from the skin-grafting, and no adverse effects or only mild soreness at the graft site is anticipated.

No or minimal adverse effects are expected from transferring a human immune system (humanisation) of these animals other than the transient discomfort from the injection.

2) For objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?"

Treatment with alemtuzumab has been shown to cause either no or very mild effects when given to hCD52Tg mice.

From the literature, we expect no or only very mild effects from from treatment with cladribine.

IL-2 is a naturally occurring chemical messenger (or "cytokine") and is not known to cause any adverse effects in treated mice when given at low doses.

Administering alemtuzumab (and other immune-depleting drugs such as cladribine) and IL-2 will require the mice to have an injection which may cause transient discomfort at the injection site.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

For objective 1 "Are expanded human CD8 Tregs suppressive in vivo?"

- Mild - 25%
- Moderate - 75%
- For objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?"
- Mild - 90%
- Moderate - 10%

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals to address objective 1 "Are expanded human CD8 Tregs suppressive in vivo?" because this is the only way to assess the full suppressive capability of these cells, including how they migrate into different tissues. Whilst experiments done in the laboratory in test tubes and petri dishes (in vitro) are useful, and will be used as much as possible throughout the project, they can not mimic cell to cell interactions that take place in a tissue/organ environment over time. For this, experiments in living animals (in vivo) are needed.

We need animals to address objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?" because it is not possible to mimic immune depletion and recovery over time in the laboratory. Furthermore, it is not possible to assess the behaviour of IL-2 within the body in a "petri dish".

Which non-animal alternatives did you consider for use in this project?

There is no non-animal alternative to address objective 1 "Are expanded human CD8 Tregs suppressive in vivo?"

To address objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?" we did consider going straight to a small clinical trial in humans. We considered this because low dose IL-2 is being given to human patients in other clinical

settings and is well tolerated. However, as described below, we concluded that this is not appropriate.

Why were they not suitable?

We did not think it was appropriate to go straight to a human study to address objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?", because, despite low dose IL-2 being safe in humans in other clinical settings, from our extensive in vitro work, we have concerns that it may cause problems after alemtuzumab or other such immune-depleting drugs.

Low dose IL-2 normally only expands Tregs. Other immune cells (called T-cells) also express the receptor for IL-2, so can respond to it. But their level of receptor expression is much lower than that of Tregs, so they are unable to respond to low doses. However, we have shown (through extensive in vitro studies) that expression increases after alemtuzumab (and the same may apply following treatment with other immune-depleting drugs). It is possible therefore, that low dose IL-2 might expand up unwanted aggressive T-cells (as well as Tregs) post-treatment. In patients this might lead to a worsening of their disease. It is therefore essential that we test this novel treatment strategy in mice first.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This number has been estimated from our extensive experience of doing this kind of work. We have estimated that 250 mice will be required for "rederiving" (i.e. establishing a new colony of mice from frozen eggs and/or sperm) any further strains of mice we need for this project. Up to 2000 mice will then be used to answer the experimental questions.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During the experimental design phase we (i) sought guidance from the NC3R's experimental design assistant on-line tool and (ii) reached out to collaborators who have extensive experience of the specific animal models we are using. In addition, we have in house experience of use of low dose IL-2 to expand Tregs in vivo, and our number estimates are based, in part, on these data. Our experimental design has also been informed by effect sizes seen in vitro (particularly for our work on response to IL-2 post-alemtuzumab).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise animal usage we will:

Breed animals as efficiently as possible - attempting throughout the protocol to match breeding with experimental need as closely as possible. This will require careful experimental planning.

Throughout the project we will perform our experiments on small groups of mice (3-5 animals at a time), in pilot studies, and will review the results prior to extending our work to larger numbers/ we will adapt our work accordingly.

Share animals with local collaborators - this is particularly relevant for NSGs where there are a number of groups locally using this animal model. This is something we routinely do on our current licence.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The two mouse strains that will be used during this project (i.e. NSGs and hCD52tg) are normal in appearance. The NSGs are more susceptible to developing infections and the hCD52tg will become more susceptible once treated with alemtzumab - but this will be mitigated by keeping these animals in a near-sterile environment.

Animals used to answer objective 1 "Are expanded human CD8 Tregs suppressive in vivo?" are not expected to suffer any distress other than that caused at the time of the skin graft surgery. A transplant model is required to address objective 1, and skin grafting is the least invasive transplant model and the transplant model with least expected complications. The surgery is done under general anaesthesia and pain relief is administered. The mice are closely monitored for the few hours following surgery and until they fully regain consciousness, this normally occurs over the first 2-3 hours by which time they are usually behaving normally. Mice will then be followed closely over the next few days and weighed daily. We expect the mice to recover well from the surgery but will be watching for weight loss and any signs of over-grooming at the site of the skin graft. Mice will remain whenever possible in their social groups.

Animals used to answer objective 2 "Does low dose IL-2 increase Tregs following lymphocyte depletion?" are not expected to experience any harm, aside from the transient discomfort from the injection(s). We expect little in the way of any adverse effects from these experiments but will monitor the mice daily and keep constant weight measurements for each mouse.

Why can’t you use animals that are less sentient?

We cannot use a “lower species” for our work, that is less sentient - because we need to study an animal with a similar immune system to that of humans, and therefore we need to study a mammal.

Furthermore, the two models we are planning to use in this project - engrafting of a human immune system, and transgenic expression of the human protein CD52 - have only been established in mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals used on this project will be monitored daily for any signs of ill health or distress. Particular care will be taken after skin-grafting, with increased frequency of observation over the first few hours post-operatively, and through the use of pain medication which if need be could be provided in the way of flavoured jelly (or such-like) that can be self-administered by the mouse. Immediately post- operatively mice will be kept warm and then will be housed in cages with additional soft bedding.

Throughout our project, we will aim to improve the quality of life for our animals – for example by providing enrichment, housing mice in social groups whenever possible and by avoiding single housing (apart from in exceptional circumstances, which will be time limited).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We are aware of, and will always aim to adhere to, good practice guidelines (as given by the Laboratory Animal Science Association; LASA). For example for objective 1 "Are expanded human CD8 Tregs suppressive in vivo?" we will ensure that we follow the LASA guidelines for performing aseptic surgery (as given in the updated 2017 document) – such as writing a pre-operative plan, administering pain medication and fluids as required and most importantly ensuring that the procedure is performed by a researcher who is fully trained.

We are also aware of the ARRIVE Guidelines (version 2.0), including their reporting guidelines around the importance of including information on experimental design and statistical analyses and strategies to minimise bias and how to report in publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will remain informed about advances in the 3Rs by speaking to colleagues locally (including veterinary surgeons, senior animal house technicians etc) and by reading information sent to us by the local licencing department. We will also ensure that we stay up to date by visiting relevant websites (including that of the NC3Rs and Norecopa) and by attending relevant conferences and training events.

As a project team we will meet regularly to ensure that we are adhering to the 3R guidelines, and implementing any changes, effectively.

107. The ecophysiology of fish

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

energy, metabolism, mitochondria, behaviour, growth

Animal types	Life stages
Atlantic salmon	embryo, neonate, juvenile, adult, pregnant, aged
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The efficiency with which animal cells produce energy seems to vary widely among animals. We aim to understand why this variation persists; one reason why it might is that animals that are inefficient in their use of energy might gain a benefit of ageing more slowly.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is important to understand why the efficiency of energy use varies among individual animals (and how it might vary over time in the same animal) because this will greatly

influence their capacity to cope with changing environments. Animals that are more energetically efficient need to consume less food per day, so can cope in situations where food availability is low. They may also be able to compete more effectively for that food, or for other resources such as shelter or mates. However, if this greater efficiency comes at a cost of greater damage to their tissues, they may age faster (or have to invest more in repairing tissue damage). This could have consequences for their lifespan, and hence for the optimal age at which they should start to reproduce. Therefore not all environments are likely to select for the same outcome to this dilemma, so producing animals with different levels of energetic efficiency. This is of fundamental biological interest but also is of applied significance, since it should influence our approach to selecting animals for captive breeding and reintroduction programmes as well as any agricultural or fish farming breeding programme.

What outputs do you think you will see at the end of this project?

This project should generate many new findings related to the links between metabolic rate, energy efficiency and performance in animals. The results are also likely to show how the optimal energetic efficiency in animals depends on the environment in which it is living. The results will be presented in the form of scientific publications and seminars, together with articles and talks aimed at a more general lay audience.

Who or what will benefit from these outputs, and how?

This is a fundamental science project that aims to provide knowledge on how animal cells optimise the supply of energy for the body. It does not have a directly applied aim, but the information generated will be relevant to those involved in selective breeding programmes, whether for food production or for conservation, since it will highlight whether the importance of maximising energy efficiency depends on the environmental conditions.

How will you look to maximise the outputs of this work?

The aim will be to publish all valid experimental results, regardless of the size of the observed effects or whether these supported the original hypotheses. We will aim to promote successful new approaches, both directly by using these approaches in collaborations with other research groups and indirectly by describing these approaches in our scientific publications. Our research group has a high profile and our previous research has been highly cited, so we would expect that we would not have difficulty in disseminating our new research to the scientific community.

Species and numbers of animals expected to be used

- Other fish: No answer provided
- Zebra fish (Danio rerio): 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

There is pronounced variation among individual freshwater fish in both their behaviour and their physiology, thus making them ideal for this kind of study. Moreover they live in simple environments, the essential features of which can be replicated in the laboratory; this has the combined benefit of reducing stress on the fish while generating results that are applicable to the real world. All life stages will be used since the study involves examining the impact of the environment experienced in early life on the performance of the animal in adulthood.

Typically, what will be done to an animal used in your project?

Experiments will typically involve anaesthetising the animal up to a maximum of 20 times, but more usually 2-3 times, in order to measure/photograph it. In laboratory experiments, fish will generally be randomly assigned to treatment groups that differ in an environmental variable such as the temperature or water flow in which they are living, or the composition of their diet. After a period of time of 2-4 weeks (rarely 2-3 months) their metabolic rate or swimming performance may be measured, and the fish humanely killed in order to collect a tissue sample for measurement of cellular function. In a small number of experiments fish may receive an injection of a substance designed to allow measurement of the rate of protein synthesis or rate of ageing; each fish will typically only receive one injection. In field experiments, wild fish will be captured, anaesthetised and measured and then returned to the site where they were caught. None of the procedures will involve surgery.

What are the expected impacts and/or adverse effects for the animals during your project?

The fish used in this project will only be subjected to benign procedures such as brief anaesthesia (typically lasting less than 3 minutes, for purposes including measuring and/or photographing), manipulation of environmental conditions (e.g. diet, temperature) or measurement of metabolic rate or swimming performance. At most they are expected to experience transitory mild pain when being given an injection.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the severity to be Mild for all animals.

What will happen to animals at the end of this project?

- Killed

- Kept alive
- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The research programme described here addresses questions about the behaviour and physiology of whole animals in response to their environment and so the objectives cannot be met without conducting field and controlled laboratory experiments using animals.

Which non-animal alternatives did you consider for use in this project?

It is not possible to conduct studies in this area using non-animal alternatives.

Why were they not suitable?

There are no means to study the behaviour and physiology of animals in response to their environment using non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Both field and laboratory experiments will be designed to minimise the number of fish used, based on previous experience and previous published research that has used similar approaches. The numbers appear large because in some cases large numbers of fish will be anaesthetised in order to measure population differences in the growth rates of fish living in different environments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Both field and laboratory experiments will be designed to minimise the number of fish used. These experiments will combine measurements across a broad range of traits to reduce the total number of fish used while maintaining high statistical power.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

These experiments will combine a broad range of measurements to obtain the maximum information from each fish and so reduce the total number of animals used while maintaining high statistical power.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Freshwater fish exhibit pronounced variation among individuals in their behaviour and physiology, thus making them ideal for this kind of study. Moreover they live in simple environments, the essential features of which can be replicated in the laboratory; this has the combined benefit of reducing stress on the fish while generating results that are applicable to the real world. None of the protocols that will be used in this project should cause more than very transient pain.

Why can't you use animals that are less sentient?

The aim of the project is to examine the relationships between the behaviour and physiology of the animal and its performance in contrasting environments, and so fully functioning juvenile or adult animals must be used.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are continuously refining our husbandry regimes and scientific procedures to minimise any welfare costs of the experiments (an example being that we now sometimes avoid the need to anaesthetise a fish by weighing it in a container of water).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The relevant Home Office ASRU Advice Notes (e.g. 03/2015 Re-homing and setting free of animals; 02/2016 Working with animals taken from the wild), the ARRIVE guidelines, and Buchanan, K.L. et al. (2012) Guidelines for the treatment of animals in behavioural research and teaching. Anim. Behav. 83: 301-309).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive regular updates on advances in the 3Rs from my establishment's AWERB, NVS and NTCO, who point me in the direction of training events (e.g. regular experimental design courses), workshops (e.g. the RSPCA fish welfare meeting) and new scientific papers describing methodological or husbandry advances.

108. Immune mechanistic models of autoimmune disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Autoimmune Disease, Adoptive Transfer, Immune Cells, Therapy, Inflammation

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project’s aims and objectives are to evaluate therapeutic entities in in vivo mechanistic models, which are based around key aspects of immune mechanisms involved in human autoimmune diseases, including graft-versus-host disease and transplantation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Although many advances have been made in recent years in our knowledge and understanding of human autoimmune disease in identifying cell types and mediators

involved in the mechanisms driving the disease process, a great unmet need for novel therapeutics that treat and cure autoimmune diseases still persists. This project will address some important immune mechanisms involved in human disease and the mode of action of novel therapeutics in modifying these mechanisms.

What outputs do you think you will see at the end of this project?

This project will address some important immune mechanisms involved in human disease and the mode of action of novel therapeutics in modifying these mechanisms with the aim to develop new therapies for patients with severe autoimmune disease.

This project will also aim to publish and share novel findings with the immunology community.

Who or what will benefit from these outputs, and how?

This project will address some important immune mechanisms involved in human disease and the mode of action of novel therapeutics in modifying these mechanisms.

Short term outputs of this project include a broader scientific understanding of immune pathways involved in autoimmune disease and the mechanims of action of certain novel targets and therapeutics.

However, of note is that novel therapeutics will in average require 10 years from an idea all the way through clinical development and onto the market to benefit patients with severe autoimmune diseases.

How will you look to maximise the outputs of this work?

We will have regular collaborations with scientific key opinion leaders in academia and industry with the aim to further advance our common understanding and accelerate the development of our therapeutics. We will also endeavor to publish key learnings in peer reviewed paper and scientific conferences.

Species and numbers of animals expected to be used

- Mice: 9000
- Rats: 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The immune system of rodents (mice and rats) shares many similarities to the human one. Mechanistic models in rodents can be used to research novel pathways and the mode of

action of novel therapies in models that translate into clinically relevant mechanistic models.

Typically, what will be done to an animal used in your project?

Animals may undergo procedures involving injections. The animals may experience short term discomfort caused by the injection. For all procedures anaesthesia will be used where appropriate and animals will be carefully monitored to ensure that discomfort to the animal is limited. All procedures have been ethically reviewed and all animals undergoing procedures will be well looked after by trained staff that work closely with a veterinary surgeon. At the end of each experiment all animals will be humanely killed, by a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

All short-term mechanistic models in this project are generally not expected to cause any signs of disease. Animals will be closely monitored and any animals displaying prolonged signs of pain and discomfort will be humanely killed, by a Schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity is between mild to moderate with most animals in the mild category.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

At present, there are no alternatives, as in in vitro technology or computer modelling and simulations that can replace the requirement of the use of animals. Autoimmune and inflammatory processes are complex and involve many different cell types in the context of a microenvironment which cannot be reproduced in in vitro models. A better understanding of all of the components involved in these processes will help in establishing viable alternatives in the future.

Furthermore, the regulatory authorities for the development of drugs state it as a requirement that novel drugs are tested in vivo for their pharmacological activity, efficacy and safety.

Which non-animal alternatives did you consider for use in this project?

Wherever possible, in vitro cell based assays will be used to study functional responses of immune cells in isolation or combination. And an up-to-date knowledge of the literature and latest technology will help replace in vivo experimentation wherever possible.

This includes the following databases for alternative methods:

- The John Hopkins Centre for Alternatives to Animal Testing (http://altweb.jhsph.edu)
- Animal Welfare Information Centre (http://awic.nal.usda.gov/)
- European Centre for the Validation of Alternative Methods (http://ecvam.jrc.it/index.htm)
- Fund for the Replacement of Animals in Medical Experiments, FRAME (http://www.frame.org.uk/)

Why were they not suitable?

Autoimmune and inflammatory processes are complex and involve many different cell types in the context of a microenvironment which cannot be reproduced in in vitro models. A better understanding of all of the components involved in these processes will help in establishing viable alternatives in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are based upon the typical n numbers required in an individual experiment and the number of experiments likely to be performed over a 5 year period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Optimum group sizes have been established for all protocols and are generally between 8-10 animals per group. These numbers are under continuous review, which take into

account the experimental variability and disease incidence to ensure statistical empowerment and meaningful and interpretable results.

A close collaboration with our Statisticians has led to a reduction in numbers of animals used for protocols which are used for projects on a routine basis.

Experimental data will be analysed using appropriate statistical tests. Experience with the experimental protocols will be applied, to ensure the appropriate group sizes are used to identify any statistically significant differences between the groups whilst minimising the numbers of animals undergoing the protocol.

Wherever possible, multiple readouts will be carried out and combined into one experiment to maximise the results obtained and reduce the number of animals used overall.

Bioimaging and multi-coloured flow cytometry, including the use of the CYTOF, will enable longitudinal studies in one animal over time and more complex readouts being combined.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

A significant proportion of our drug discovery process involves in vitro assessment of compounds using cells and cell lines, with many thousands of potential drugs being screened to identify the most promising compounds/antibodies. However, in order to study complex inflammatory responses, further testing is required in animals in which disease symptoms have been induced.

To ensure the fewest number of animals are used, only the most effective compounds/antibodies that have been pre-screened for activity in vitro will be examined in animals. Only a limited number of these compounds/antibodies will be evaluated in this project. and those molecules will have been previously evaluated in simpler in vivo mechanistic models.

An initial pilot study consisting of a single high dose group plus control may be conducted initially to determine efficacy prior to larger multi dose group studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All new molecules will have undergone in vitro profiling prior to testing them in vivo. Pharmacokinetic studies will have been carried out to ensure that suitable dosing regimens are applied to produce the required exposure to show in vivo efficacy.

In our experience, adverse drug reactions have generally been found to be rare (<1%) with in vitro screening assisting in the identification of entities that are likely to be toxic. All protocols are under constant review, so that any new data from the scientific and clinical literature can be considered in the development and optimisation of the animal models.

Within each protocol, we aim to limit animal suffering through a careful choice of stimuli and adjuvants used. Distress scoring sheets are in place for some protocols and employed to help monitoring of the animals.

Why can’t you use animals that are less sentient?

Rodents (mice and rats) are the most appropriate species to use to study immune mechanistic responses and immune inflammatory responses. The use of transgenic mice leads to more robust readouts especially when following particular cell populations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

A greater knowledge of our established models allows us to select the most appropriate model to use to study a particular mechanism implicated in disease. Distress scoring sheets are used to monitor the severity of adverse events and experimental data are under constant review to determine that the correct level of in vivo immune activation is achieved with minimum stress to the animal. Humane endpoints are employed to limit animal suffering.

As we learn more about the models in terms of response rates and clinical signs, we continue to refine protocols with updated animal welfare sheets, adjusted protocols and endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance will be taken from organisations such as the nc3Rs and LASA.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously review and learn from the literature and wherever possible incorporate any new advances in our 3R strategy.

The nc3rs.org website is a useful source of information regarding advances in the 3Rs which can be consulted for updated advice. Additionally advice will be sought from the NVS.

109. Mechanisms of cardiometabolic disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Obesity, Diabetes, Lipids, Lipotoxicity, Cardiometabolic

Animal types	Life stages
Mice	adult, neonate, juvenile, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine how obesity causes metabolic complications.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity is one of, if not the leading cause of multiple major diseases. These include diseases traditionally classed as "metabolic" such as diabetes, and cardiovascular diseases such as heart attacks, stroke, and heart failure. Increasingly, however, obesity is also recognised to be a significant risk factor for diseases such as cancer, asthma, kidney

failure and arthritis. Obesity can also increase the risk of severe complications and death from infectious diseases such as Covid 19.

However, we know that what links obesity to diseases is not the weight itself. If you walk around carrying an 80 kg backpack all day, you do not get diabetes - you become physically fit. We believe that what connects obesity to metabolic diseases is primarily a failure in fat tissue itself. Each individual has a genetically and environmentally determined limit on their ability to store fat safely. When that limit is reached, fat begins to accumulate in other organs and cell types. Scientists have now found that this accumulation happens in almost every organ and cell in the body they have studied, from the liver and muscle where it causes insulin resistance which leads to diabetes, to the immune system, where it can increase our risk of asthma and arthritis. We aim to study how fat tissue stops working and when it does, how to either prevent lipid accumulation in other organs, or make that lipid accumulation less damaging.

In an ideal world we would combat the diseases caused by obesity by treating obesity itself. However, despite decades of work by academics and the pharmaceutical industry, it has turned out to be nearly impossible to design drugs to reduce food intake and body weight. Given that we can't make people lose weight, trying to reduce the damage obesity does to our bodies is an essential approach to reducing the disease burden (as of 2016 the WHO estimated that 1.9 billion adults are overweight worldwide). It is estimated that obesity and its associated cardiometabolic diseases will cost the UK economy 50 billion GBP per year by 2050 (www.gov.uk). Furthermore, obesity has a tremendous quality of life cost, increasing the likelihood of developing diabetes 5 fold, some cancers by 3 fold, and high blood pressure, a major risk factor for heart disease by 2.5 fold. Overall, obesity was estimated to account for 23.1% of all deaths in the UK as of 2017.

What outputs do you think you will see at the end of this project?

We have **six objectives.** Our first three focus on how fat tissue (also known as adipose tissue) stops working. Healthy fat tissue **a)** buffers the amount of fat (known as lipid) in our blood to allow us to safely cope with eating different types of food; **b)** stores nutrients for the long term; **c)** secretes a mixture of proteins that regulate all aspects of our body's function. When fat tissue stops working correctly, these functions break down and the lipid that should go to fat tissue ends up in other organs like the liver and muscle where it stops insulin working, leading to diabetes. It can also cause heart failure by poisoning the heart, and it can also cause inflammatory diseases like arthritis by poisoning the immune cells.

In our first three objectives, we will determine **a)** the mechanisms and genes responsible for causing a breakdown in lipid buffering; **b)** determine how our bodies wound healing systems, which lead to scarring, get inappropriately activated in fat (and other tissues) and stop it working; **c)** validate how three genes that have been identified as possibly limiting adipose tissue function in humans actually stop fat tissue working.

These three objectives are essential as all three represent understudied or new processes/genes that may link obesity to metabolic complications and thus provide new drug targets.

Objective 4 focuses on how to combat the metabolic defects of failed fat tissue. As mentioned above, when fat fails, lipid goes to other organs. If we can reroute this fat to organs which can burn it (muscle and a specialised thermogenic organ called brown fat), we can stop it from accumulating in other tissues that cannot burn it. However, if we are to send fat to organs that can burn it, it is essential to make sure we can also actually activate their fat burning systems, otherwise they will simply fill with fat and stop working themselves.

Turning on fat burning systems is an attractive approach, as it potentially allows treatment for a collection of "cardiometabolic diseases" including diabetes and heart failure. In addition to treating these diseases, it may also cause weight loss through increasing energy expenditure.

Objective 5 focuses on how lipid accumulation in other organs causes toxicity. Under this license we will look at how the toxic accumulation of lipids in organs and cell types, including liver, heart, muscle, pancreas, and the immune system, leads to a wide range of obesity-associated metabolic diseases.

Objective 6 is a more specific objective to validate a better model of human liver disease progression. Liver disease (known as NAFLD) goes through several stages. The mildest is called "non-alcoholic fatty liver" (NAFL). The next stage is called "nonalcoholic steatohepatitis" (NASH). NAFL is characterised by inappropriate fat accumulation, whereas NASH has fat accumulation, inflammation and liver scarring. We currently have no good models to study how NAFL turns into NASH. Three dietary models of fatty liver disease have been selected to best mimic human NAFLD, and in particular to look at how NAFL turns into NASH. These models have been selected based on historical human and animal data from thousands of experiments. However, the data has been collected over the years and is patchy and non-standardised. We will test the three best candidate models to determine which is the best.

Objectives 1-5 will primarily produce scientific papers as their main output. In addition, we have links with industry and will look to move the results from these objectives into early-stage clinical trials and biomarker discovery. Biomarkers and things we can measure in the blood that tell us about the stage of disease help decide when and how to treat people, and provide information on whether drugs work. We disseminate our data at conferences and via web based media outlets predominantly targeted at the scientific community. In addition to scientific outputs, we also engage with the public and produce lay science pieces.

Who or what will benefit from these outputs, and how?

In the short term, the primary beneficiaries will be the scientific community. The immediate beneficiaries will be those working in the field of metabolism. However, given the range of diseases obesity is a risk factor for, we expect our research to be of benefit to the fields of immunology, cardiovascular disease, hepatology (the study of the liver) and cancer.

Our work will help to inform human clinical trials in both academia and industry. In the longer term, we expect our research to benefit the pharmaceutical industry, where the genes and processes we identify will provide new targets for creating drugs to combat obesity-associated diseases. We have links with the university technology transfer office to maximise the value of our work by patenting it where appropriate.

In particular, **objective 1** may also benefit nutritionists and dieticians. This objective strongly focuses on the impact of the timing of nutrient usage and how it can worsen or improve metabolic disease. As such, it may lead to personalised diets for people with different macronutrients at different times of day (e.g. fat in the morning for some people, carbs for others and vice versa).

Finally, if our work leads to new treatments for the metabolic complications of obesity it will benefit the general public.

For objective 6, the overall benefits will be the same. However, the benefit to the pharmaceutical industry will be short-term rather than longer term as we expect an immediate adoption of the chosen optimised model of fatty liver diseases for the development of new drugs.

How will you look to maximise the outputs of this work?

We have many national and international collaborators with whom we share our data and ideas.

We will publish our work in open-access publications. In addition to traditional dissemination routes, we have a website where we post videos regarding our work, in particular technical approaches we have developed for studying metabolism.

Furthermore, we actively engage with the public via lay science pieces and specific web-based media and podcasts.

Concerning the publication of unsuccessful approaches, this perhaps misses the point of science. One of our most successful publications in terms of citations, describes a paradoxical lack of phenotype in a mouse - sometimes the absence of "success," i.e. finding negative results - can be more important than finding many significant changes. Indeed we are currently preparing a manuscript comprised of exclusively non-significant (“unsuccessful”) results which we think will be of great importance to the field.

Species and numbers of animals expected to be used

Mice: 22500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using weanling and sexually-mature adult mice up to 15 months of age. This allows us to study cardiometabolic diseases, which are now recognised to impact all ages but become particularly prevalent in middle age. These are the least sentient species suitable for studying metabolism, which involves the complex interplay between many organs. Moreover, there is a large amount of data regarding specific strains of mice and how they develop metabolic diseases, making them suitable to study the function of genes that affect specific aspects of human cardiometabolic diseases.

Typically, what will be done to an animal used in your project?

We will be studying mice that develop metabolic disease. These will typically be induced by feeding a modified diet (e.g. high-fat or high-fat/high-sugar diets). Animals may remain on these diets for up to 14 months (from weaning at 3-4 weeks) but typically for a much shorter duration, and this would be expected to lead to obesity, insulin resistance and other obesity-associated metabolic complications, including liver disease, reduced heart function, and inflammation.

We will use genetically modified animals to work out the mechanisms that connect obesity to metabolic diseases. This will enable us to tell which genes/processes predispose us to develop metabolic disease or alternatively protect us against it. We know that temperature is a critical variable for controlling mouse metabolism and we may house mice at different environmental temperatures to control for this. Equally, we will use altered temperatures to investigate how mice make heat.

In a small number of animals under this license (less than 5 percent), we will perform a bone marrow transplant to study how the immune system affects metabolic disease. We will need to irradiate mice and give them bone marrow from another mouse.

In addition, an animal may undergo one or more of the following procedures, but a typical animal will experience no more than five of these:

Mice may receive drugs to alter their metabolism or to delete genes selectively at specific times or in particular organs, either through injections (these can be into a vein, into the body cavity, under the skin or directly into an organ), or orally (by mouth via a tube inserted into the stomach or in drinking water). Not all animals will receive such drugs and the volume and number of injections used to administer drugs will be the minimum needed.

Mice may be given substances known as tracers which allow us to understand more about their metabolism. Tracers are substances that allow us to study a metabolic process but do not affect it. For example, we can give a mouse a glucose tracer to determine if it has insulin resistance in its liver, muscle, or both. Not all animals will receive such substances. We give tracers by injection into their body cavity, into their veins or under their skin, and/or orally (in their food, drinking water, or via a tube into their stomach). Typically a single tracer injection is sufficient with/without administration of the tracer in drinking water, but some mice may receive up to 4 injections.

We may house mice alone for periods of time (typically no more than 3-4 days, but on occasion up to 4 weeks). This is necessary to measure the amount of energy they are expending, their activity and food and water intake, and to determine what nutrients they are burning and when. We will also singly house mice for food intake assessment. This can take as much as 2-3 weeks to get a reliable measure of food intake as mice can fluctuate their body weight by +/- 5% in 24 hours under free living conditions (equivalent to 4 kg in an average human!). As such their food intake is very variable over the short term but becomes more stable in the longer term.

Mice may be scanned using a non-invasive imaging method (e.g. TD-NMR) to examine their body fat percentage. Animals are briefly restrained (usually less than 3 minutes) in a plastic tube. This is both painless and harmless.

Some animals will be anaesthetised for less than 1 hour so that we can image their organs (usually heart or liver) using either ultrasound or MRI. We need to anaesthetise the mice in order to obtain clear images without the animal moving around. No animal would be expected to undergo this more than 6 times in their lifetime with at least 1 week between each imaging procedure.

At the end of the experiment, each animal will be killed using the most humane method that is possible, that does not prevent us from obtaining good scientific data.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals developing features of metabolic disease will show a range of adverse effects, including: obesity and insulin resistance, inflammation, tissue fibrosis and reduced heart function. They may experience some general discomfort associated with their increased body weight and become lethargic. Conversely, some specific diets (particularly to cause fibrosis in the liver) can cause mice to lose a large amount of weight. This weight loss happens in all mice and is not in itself a sign of illness. As weight loss is not a good sign of ill health when mice are on these diets we have to carefully monitor animals in other ways to ensure that they are not unwell. None of our interventions are expected to cause pain. Some genetically-modified animals might be expected to show similar adverse effects, whilst others would be protected from the impact of metabolic disease.

Animals undergoing bone marrow transplants may be unwell for several days after the irradiation, eat less, and thus lose weight. They are expected to start to recover body weight after 14 days and fully recover by 4 weeks.

Mice are not expected to experience any lasting harm from injections per se. Tracers would not be expected to cause any adverse effects. Drugs which alter metabolism may lessen or worsen the adverse effects associated with metabolic disease, but we will test them to make sure they do not cause unwanted side effects that are harmful to the mice.

Single-housing can be stressful for mice, but we will minimise the time each animal is housed alone. Where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing.

Animals anaesthetised for imaging/implantation of microchips (e.g. for sending body temperature to a receiver) will feel groggy as the anaesthetic wears off but will experience no pain or lasting harm. The imaging processes themselves are painless and harmless.

Non-invasive imaging (e.g. TD-NMR) for body composition analysis requires restraint, which can be stressful to mice, but causes no other pain or harm to mice.

Expected severity categories and the proportion of animals in each category per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

97% mild

3% moderate

What will happen to animals at the end of this project?

Killed

Kept alive

Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cardiometabolic diseases involve the interplay between many different organs. For example, the development of diabetes as a result of obesity mostly involves dysregulation of food intake control by the brain, a loss of fat storage by the fat tissue, the accumulation

of toxic fat (known as lipid) species in the liver, muscle and the immune system that prevent insulin working. In turn, the beta cells, the cells in our pancreas that produce insulin, over-secrete insulin to counteract this "insulin resistance". The beta cells can only produce so much insulin and as insulin resistance progresses they ultimately fail. Such a system cannot be modelled in a dish in the lab (in vitro). Instead, it has to be studied in a mammalian system such as the mouse.

Which non-animal alternatives did you consider for use in this project?

We look to use animal alternatives where possible. We use cell culture models (cells in a dish in the lab that allow us to study aspects of how specific organs work) to look at specific mechanisms that occur in isolated cell populations. Results from these experiments can both be informed by our animal work and help us to design better animal experiments.

In addition, we have developed two sophisticated culture systems to help us to study fat and liver.

Firstly, over the past 5 years, our laboratory has been developing a human stem-cell based adipocyte culture system. This protocol takes us in a stepwise manner from stem cells through to fat precursors,

and then ultimately mature fat cells. Importantly it captures all the known developmental stages that the stem cells go through before ultimately becoming fat cells. The stepwise manner of our protocol makes it a powerful tool to probe genes regulating fat cell formation.

In addition to the work on stem cells, we have also developed an improved in vitro (in petri dishes) model of human hepatocytes (the primary cell found in the liver that performs its metabolic functions like controlling blood glucose levels) culture. A long standing issue in the field of liver disease has been the lack of good in vitro models, with even primary isolated hepatocytes losing many of the features they show when still in the mouse. In culture, hepatocytes rapidly regress from being mature hepatocytes and instead come to resemble fetal-like (similar to the immature hepatocytes found in developing babies while still in utero) cells within hours.

We have collaborated with a company that uses special liquid handling systems and complex three- dimensional biological “scaffolds” to produce synthetic organs. We have worked with them to create a model of human liver that includes both the hepatocytes and the other liver cells (Kupffer cells, the primary immune cell of the liver and another liver support cell, called the stellate cell). The results from this system were remarkable – producing a set of conditions which kept hepatocytes in almost the same state as we see in vivo.

Why were they not suitable?

While cellular models, even sophisticated ones such as the "organ on a chip" liver model (a tool that aims to reproduce the biology of an organ in silico), can address specific questions, they cannot allow the study of the complex interplay between multiple organs that leads to metabolic disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include, e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers are primarily based on the level of work over the previous two licenses and the amount of funding we have in place and expect to use to fulfil our aims and objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Metabolic disease has a complicated and lengthy disease progression. When we try to study it using diet-induced obesity, early stage events will occur in days, if not hours, of diets being changed, later stage events manifesting over weeks, and long term events that take months to appear. Given that many events happen at many times, perhaps the most critical question for reducing animal usage is to determine the correct length of time a mouse should be fed a high-fat diet for before we begin studying it.

For example, one specific lipid in the liver of mice called arachidonic acid will reset its levels within 24 hours of a change in diet and then remain constant for 3 months. This lipid is a key regulator of inflammation. As such many genes use it to make signalling molecules and there is a sophisticated system is in place to defend its levels. If we work on genes that regulate arachidonic acid levels, then the most suitable length of time to feed a diet to a mouse may be as little as 24 hours. Conversely, fibrosis is the cumulative build-up of scar tissue due to inappropriate activation of wound healing processes. It may take a period of as much as 9 months of high-fat feeding to develop measurable fibrosis in some organs.

To address these questions we have set about modelling the development of obesity associated metabolic complications to allow us to select the optimum length of interventions for studying specific disease processes. We have built several time courses of responses to different diets, which gives us much better information for selecting the length of our intervention.

In addition to optimising the conditions, we carefully consider which control groups are necessary in order to address the scientific question as we design individual experiments, and we use power calculations to ensure that we are using an appropriate number of animals.

We consider carefully how to get the most out of each animal we use. For instance, many of our animal studies involve the generation of models of metabolic disease (often reflecting a human condition such as diabetes, obesity or fatty liver disease). The nature of these diseases is such that they affect many organs of the body. As such, if we plan an animal study designed to probe the effects of diabetes on the liver, for instance, we think carefully about which other organs researchers in the laboratory can work on, such as heart and adipose tissue, and harvest these from the same animals. This reduces the number of animals used overall, increases the amount of data obtained from a single animal, and allows us to examine links between different tissues/organs by combining data from the same animals, thereby enhancing the quality of the information produced.

For several specific techniques we have made changes that enable us to reduce the number of animals we need in experiments.

For body composition we have performed a series of extensive trials to optimise the conditions under which body composition is performed. These allow a low variance in data to be obtained. Variance is how much the data is spread. To be confident a result is accurate, we need either data with low variability or to use lots of mice. By reducing variance we improve data quality, decrease the need for repeat measurements, and reduce the number of animals needed per group. Additionally for euglycaemic-hyperinsulinaemic clamping (the best method for measuring insulin sensitivity), we have comprehensively reworked our protocol to use smaller infusion volumes by using smaller tubing and better mixing taps. This has reduced the time the procedure takes, improved data quality, and reduced animal usage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have increasingly begun to use pilot studies. These were initially used under our previous license to check the safety of drugs in a small number of animals before using them in a larger cohort. However, researchers rapidly realised the benefit in terms of being able to assess the efficacy of these drugs from the pilot animals.

We employ a designated member of staff who oversees our animal program to ensure the breeding of colonies and experimental design is optimal, and animal wastage is minimised. We routinely cryopreserve all our lines to avoid "tick over colonies" wasting mice and also prevent the phenotype of the mice from changing due to being bred for many generations.

We collaborate widely with other groups across the UK, Europe and the rest of the world and regularly ship samples we have banked to these collaborators for their own purposes, maximising the benefit from previously conducted studies.

We are actively engaged in pursuing data science and artificial intelligence based approaches to the study of metabolism. We regularly reanalyse our old data sets with new collaborators who are experts in the field of data science. As approaches move on, more sophisticated or new ways of interpreting gene expression, lipid and protein data are developed. As such, our existing data may lead to exciting new discoveries and new publications with no need for extra mice.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice.

We will typically use a mixture of genetically altered (we have deliberately introduced some DNA into them), spontaneous genetic mutant animals (e.g. the ob/ob mouse was found in laboratory colonies as a mouse that was enormously fat - it has a single DNA base pair that has gone wrong) and "wild-type" animals (we have not genetically altered them, nor do they have any known mutations). The majority of our genetically modified animals are congenic, possessing the DNA we have introduced into them in all cells from conception. We also work with a number of mice with pieces of DNA called “loxP sites”. These loxP sites allow deletion of the gene in one or more organs by expressing a gene called a recombinase (known as Cre) that deletes anything between the loxP sites. The deletion of the gene is usually caused by making the Cre have the same expression as a gene found in a specific tissue (e.g. albumin, the most common protein in the blood, is produced only in the liver). We can make the Cre inducible. This means it is normally not active, but if we give the mouse a drug, it turns on the production of the Cre recombinase and deletes our gene of interest. In some cases we will use viruses to express the Cre. The genes that will be altered will be chosen to address our objectives. They will largely fall into four categories: Those affecting adipose tissue function, those affecting energy expenditure (thermogenesis), those affecting lipid quality or quantity in organs, and finally those are leading to alterations in fibrosis. Importantly, all of the major areas we are researching are involved in human diseases which cause minimal pain. Even end-stage liver and heart failure are characterised by signs such as weight loss (liver failure) or shortness of breath (heart failure). Therefore, we do not expect our genetic mutations in and of themselves to cause any pain, suffering or distress. The development of metabolic disease is slow, so we will be able to monitor for and kill mice before more serious harms manifest.

We will use two primary routes to induce obesity and study its complications. The first is the use of modified diets that make mice obese and insulin resistant, as well as causing diseases such as liver and heart disease. These are usually high in fat, contain refined carbohydrates, and we may also add sugar to the drinking water. It is notable that human fast food tends to be high in fat and refined carbohydrates, and is often accompanied by sugary drinks, making this a depressingly accurate model of the "western" food intake patterns driving the global obesity epidemic. High-fat diets are preferred by mice when given a choice between them and chow. They can lead to a greasy coat but generally cause no pain, suffering or distress.

In addition to obesity we may use diets to study specific aspects of lipid metabolism, where they contain different types and/or quantities of fat. Equally we may use diets to induce fibrosis that lack specific micronutrients (such as amino acids or vitamins). These may cause weight loss, but the weight loss is not caused by the fibrosis, but due to the fact the diet is not as tasty to the mice (the opposite of the high-fat diets).

The second approach for causing obesity will be to use genetically altered animals that model specific aspects of cardiometabolic disease. The most commonly used animals in our laboratory are the spontaneous mutant ob/ob and db/db animals that lack leptin hormone or its receptor respectively. Leptin tells our bodies we have enough fat. When mice lack leptin, they will keep eating as they think they need to store fat, even though they have large amounts already. A loss of leptin causes massive obesity due to a high food intake. The ob/ob model is useful as it causes obesity without the need for the feeding of altered diets. This can make the ob/ob particularly suited for studies where manipulating dietary lipid composition and content would confuse the interpretation of our results. The ob/ob mice are extremely obese and need some special care. They need to have larger mouse houses. Furthermore, they may develop diabetes and drink and urinate more, requiring more frequent cage changes to stop them from developing sores on their body.

In terms of phenotyping, we use several major types of analyses. Firstly, we use "tolerance tests" to determine the response of mice to different nutrients and/or hormones. This involves an injection or use of a tube that goes into the mouse’s stomach to deliver a fixed amount (known as a bolus) of fat, carbohydrate, protein or insulin, followed by the collection of serial blood samples, usually from the tail vein of the mouse. We take serial samples to see how different metabolites in the blood of the mouse change in response to the bolus. We have a dedicated team of trained technicians who conduct these tests routinely and are able to take the minimal blood volumes that are scientifically necessary. As they perform these tests for multiple groups they are highly experienced and are able to conduct these bleeds without restraining the mouse.

We also assess energy expenditure using a device called an indirect calorimeter. Calorimeters measure heat - which is proportional to how much energy the mouse is expending. We conduct calorimetry in home cages and it has a minimal welfare burden.

We house animals at altered environmental temperatures. Mice are normally housed between 20 and 24oC. We have specialised cabinets to control the temperature we house mice at. Almost all mice can tolerate temperatures as low as 4 degrees and as high as 30 degrees well. Considerable evidence suggests higher environmental temperatures make the metabolism of mice for many parameters we are interested in (development of fatty liver, cardiac function, energy expenditure) more comparable to humans. Equally, lower temperatures can allow us to study processes such as heat production in more detail. The cabinets we have are able to keep the temperature within 1oC of the target temperature preventing dangerously high or low temperatures from occurring. Rarely some genetically altered mice will not be able to survive in the cold. We carefully monitor animals from strains we have not studied before when we place them in cold cabinets for the first week to make sure they are able to tolerate low temperatures.

Finally, we have a number of specialised techniques including clamping (a sophisticated technique for assessing insulin sensitivity that provides better and more detailed information than tolerance tests) and thermogenic capacity (a technique for measuring the most energy a mouse can expend) measurements. In the literature these are both routinely conducted on conscious or unconscious mice. We perform them at the end of the experiment on unconscious mice that have been given anaesthetic. The mice are killed before they wake up meaning they suffer as little pain and distress as possible.

Why can’t you use animals that are less sentient?

We use less sentient animals where possible and have published using flies to study specific research questions. However, two of our major focusses in this license are on adipose tissue function and thermogenesis (the process of heat production). Flies do not possess adipose tissue. Furthermore only mammals and birds possess the major thermogenic organ brown fat. As humans are mammals, then a mammalian system is more suitable for translation to humans than an avian system.

We do use terminally anaesthetised mice for some procedures, however metabolic disease takes years to develop in humans and months in mice. As such we need to conduct tests in either conscious mice, or using recovery anaesthesia, to track the development of metabolic diseases over time.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have made multiple refinements to our techniques over the past few years. We undergo a continual practice of reviewing our procedures in order to improve them from both welfare and scientific perspectives. Indeed, these are not separate endeavours. The researchers under the license are all aware that the higher the standard of welfare, the better the quality of the data, making them committed to all aspects of refinement.

With respect to glucose tolerance tests (a test where we administer a single large dose of glucose and then see how well the animal can return its blood glucose to normal) we have

implemented various stable isotopic forms of glucose. Stable isotopic forms of glucose are types of glucose that are biologically identical to normal glucose, but have atomic "labels" which we can detect using a device called a mass spectrometer. We can use stable isotopic glucose to determine changes in the mouse's own glucose production (from the liver) during a GTT and separate that from the glucose we have given the mouse. As such we can see if our mouse has changes in liver function, which can occur in insulin resistance, as well as more general problems dealing with a large amount of glucose. Stable isotopes are biologically identical to normal glucose and therefore provide more data from the same experiment for no more pain suffering or distress.

For clamping we have added stable isotopes allowing the harvesting of tissue (as the samples are non- radioactive). This allows molecular analyses and histology to be connected to gold-standard measures of insulin resistance (for example we can see if mice with bigger adipocytes, or more inflammatory molecules in their blood are more insulin resistant).

For calorimetry we have improved our data analysis by regressing the variable RER against change in body-weight, reducing the variance ("variance" is how noisy the data is – the noisier the data the more mice you need to be confident an effect is real) and improving the biological meaning obtainable from the data. We also singly house mice ahead of calorimetry experiments to acclimate them to the new environment before conducting the tests. Furthermore our new calorimeter is an open circuit system, with smaller sensors for things such as movement. This means we can house mice that were formally group housed close to their cage mates, with better vision of them and, because it is not a fully sealed chamber, they can also hear each other.

For altered temperature housing we use temperature chips which allow us to monitor the mice for drops in body temperature without using more invasive approaches like rectal thermometers.

For feeding of modified diets we have reduced the amount of diet added to the cage and increased diet changes to improve palatability of the food for the mice and as a by-product data consistency. High fat diets go "off" more quickly than chow ones as the fats in them can become rancid and have an unpleasant odour and taste. Furthermore, through highly precise food intake measurements we have been able to identify the importance of consistency of when animals are culled after a diet change. The replacement of old high-fat diets with new leads to a spike in food intake. This is important as spikes in food intake may change the expression of genes or cause increases in hormones like insulin in blood. By controlling when mice are culled after a diet change we reduce unnecessary noise, (and decrease variance) and reduce the number of mice we need to be sure a result is real.

Feeding of modified diets. Certain modified diets (particularly those that cause fibrosis in liver) can cause mice to lose weight. This weight loss is not a sign the mice are sick - all the mice lose similar amounts of weight and end up lean. However, this means we cannot

use weight loss as a sign mice are not well. To improve our ability to assess mice we have now included body condition scoring. We use a chart that includes pictures for reference as well as clinical signs to make sure our mice are healthy. We conduct such scoring weekly, unless mice are showing lower body condition scoring in which case we may do it more frequently.

We have now included deuterated water (D20) tracing. D20 is water which heavy hydrogen (deuterium) has replaced the normal hydrogen. When our bodies make fat from sugars they incorporate hydrogen or deuterium from water. If we give mice D20 we can detect the new fat and distinguish it from the old fat using a mass spectrometer. Alterations in how much fat our body makes occur in diseases like diabetes and NAFLD. This provides additional important scientific information with no additional welfare burden to the mice.

For administration of agents we implemented an agent safety protocol. This follows a classic 3+3 design used in phase 1 human clinical trials and involves dosing 3 animals with the drug for one week to determine the agent is safe. We are also able to collect serum and tissues from these samples to provide pilot information for subsequent larger studies.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique(https://www.lasa.co.uk/current_publications/)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (https://www.ncbi.nlm.nih.gov/pubmed/28771074).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project license (PPL) holder is subscribed to the National Centre for the Replacement and Reduction of Animals in Research (NC3R) monthly e-mail newsletter and uses the NC3R website (https://www.nc3rs.org.uk) as a resource for guidelines, practical information, links to publications and video and training materials. The PPL holder has attended the international Federation of European Laboratory Animal Science Associations (FELASA) conference. In addition the animal technicians who work for the PPL holder's institute regularly attend both Laboratory Animal Science Association (LASA) and FELASA conferences and disseminate information regarding 3Rs approaches to the institute. The PPL holder is an editorial board member of Laboratory Animals, the official

journal of both LASA and FELASA, which published advances in animal welfare and husbandry matters.

Advances in the 3Rs will be disseminated to the laboratory through the weekly laboratory meetings and through meetings with our technician core in order to have a holistic approach incorporating the license holder, researchers and technicians.

110. Developing and studying novel mouse models of glioma development and progression

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, brain, IDH, gliomas, hydroxyglutarate

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To investigate the role of specific genes, identified in human patients and in vitro models, in glioma development and progression, in order to understand the disease process and thus improve prevention, early diagnosis and treatment options.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Gliomas represent 75% of all primary malignant brain tumours in adults. They can be divided into less aggressive (oligodendroglioma and astrocytoma grade 1 and 2) and more aggressive (oligodendroglioma, astrocytoma grade 3 and glioblastoma grade 4) tumours. Because of their malignant behaviour and the fact that they cannot be cured by current therapies, they represent some of the most devastating cancers. The aim of current treatments is simply to delay tumour progression and modestly extend survival. Despite decades of refinement, this approach results in a median survival time of just 12-14 months for glioblastoma, a survival trend that has remained mainly static in comparison to other cancers. A better understanding of the underlying mechanisms is urgently needed in order to design new and successful therapeutic strategies.

Advances in cancer treatment and patient survival often follow a greater understanding of the molecular basis of the disease. Through combined genetic, biochemical and metabolic studies, researchers have begun to uncover some of the early events in the development of brain cancer. It is now understood that glioma is caused by metabolic disturbances due to alterations in a specific gene called 'IDH1' (and less frequently, IDH2). However, very little is understood about how these changes actually drive malignancy, or in which specific brain cells these alterations arise.

Our work aims to develop accurate mouse models of glioma by targeting IDH1 alterations to specific brain cells, and to use these models to look at genetic and metabolic interactions with other common changes identified in patients. Here in this proposal we aim to identify an optimal glioma model, before applying this model to study the role of two important genes, D2HGDH and CIC, in glioma development.

Overall, the work is important because it not only has the potential to significantly improve the ability of researchers to accurately study glioma in vivo, but also to identify therapeutic targets and potential new treatments.

What outputs do you think you will see at the end of this project?

Our principal output at the end of the project will be important new information on glioma development, including advances in mouse models of glioma that more faithfully recapitulate the human disease, and improved understanding of the molecular and genetic interactions between IDH1 and other common glioma alterations. The work will generate high profile publications in scientific journals and inform on new therapeutic targets and treatment options to fight these tumours in humans.

Who or what will benefit from these outputs, and how?

In the short term our findings will contribute to an improved understanding of how gliomas develop and progress, which will be disseminated to members of the scientific community through scientific publications in high impact journals and presentations at scientific meetings. Identification of a more accurate mouse model of glioma will also represent a major advance that will be of direct benefit to the whole brain cancer research community.

In the medium term, our work may suggest specific molecular targets to be used in designing new therapeutic strategies. Thus pharmaceutical companies committed in designing new treatment options for brain cancer will benefit from our work. In the longer term, we hope that the work, and subsequent clinical application of our findings by pharmaceutical companies, will improve treatments and outcomes for patients affected by glioma and related cancers.

How will you look to maximise the outputs of this work?

All the new mouse models generated by our group will initially be made available to collaborators and then to all other researchers following publication.

We aim to continue collaborating with various research groups with different and complementary knowledge and expertise, which will help to maximise the transfer of knowledge arising from the work. The findings will be communicated in open access scientific journals and at international conferences to make them broadly available prior to publication.

We do not underestimate the value of a negative result, therefore whenever possible we shall publish these in scientific journals or make them available for example though an open access platform like BioRxiv.

Species and numbers of animals expected to be used

- Mice: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to use mice because they represent the least sentient animal model that most closely recapitulates human tumour development and because their genome is relatively easy to manipulate. Our laboratory also previously developed a preliminary model demonstrating that mice are suitable for modelling glioma development. This prior work has established protocols and endpoints that can be applied in the mouse models described here. The genetic variants to be studied here are available as established mouse strains, thus avoiding the genetic manipulation and breeding required to generate these models in a different animal type.

Where possible we will use all strains on a C57BL6 pure background to avoid experimental variation and the necessity for increased numbers. Conditional genetic alterations will be induced in young adult mice (generally around 6 weeks of age). Adult mice are relevant because glioma is a disease of human adults, and because some of the genes are lethal when deleted earlier in life.

Typically, what will be done to an animal used in your project?

In a typical experiment, animals will be bred with other mutant strains to obtain the required combination of genetic alterations to allow the role of genes in specific cell types to be studied. When necessary, young adult mice will be injected with the inducing agent (tamoxifen), and then aged to specific time-points (e.g. 4, 8 months), when they will be humanely killed and samples collected and analysed. In those cases where the timeline of disease progression is unknown, mice may be aged for up to 15 months until they develop symptoms of tumour formation.

What are the expected impacts and/or adverse effects for the animals during your project?

Since animals used in the proposed project represent models of human brain tumours, some of them will develop symptoms similar to those of human patients affected by a similar disease. These symptoms can be related to the tumour being in the brain (e.g. change in behaviour, hydrocephalus) or general symptoms of sickness (hunching, loss of weight, lethargy). Based on our previous studies some mice may develop the described symptoms from 4-6 weeks after tamoxifen administration. The duration of these adverse effects may be variable, but when an animal develops symptoms such as weight loss (up to 20%), lethargy, hydrocephalus or visible tumour mass, they are humanely killed and samples collected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (65%)

Moderate severity (35%)

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In the proposed project I am planning to investigate the functional mechanisms of brain tumour onset and progression in order to improve early diagnosis and treatment. To

achieve the aim of the project I need to use animal models as they are more informative and provide major advantages over in vitro, ex vivo or in silico models:

1) Cancer is a disease arising from specific cell types within a complex multi-cellular environment, which cannot be faithfully recapitulated by homogenous in vitro cell models.

2) The genetic alteration is targeted to a specific cell population which provides the opportunity to investigate the physiological function of a gene and its pathogenic role when mutated, in the correct cell type and context.

3) Animal models allow us to explore the mechanisms of cancer development and progression from early stages and to understand the influence of different combinations of genes (which is limited by using in vitro and ex-vivo models).

4) Animal models also allow us to study the biological properties of a developing tumour in a more physiological system, including a vasculature, immune cell infiltrate, tumour microenvironment, and an intact blood-brain barrier (which are not possible non-animal models).

Which non-animal alternatives did you consider for use in this project?

For the reasons outlined above, non-animal models are not suitable for studying early glioma development. That being said, whenever possible my group conducts experiments before and in parallel to in vivo work using in vitro models, in order to provide supporting data and to test hypotheses that may not at first require an in vivo model. These approaches include pathology analyses of human brain tumour samples, and analyses of cerebral brain organoids, glioma tumourspheres and glioma cells.

Why were they not suitable?

Non-animal models can be useful in answering some questions, but are not suitable for studying tumour development in the context of a tissue. This is because cancer onset and progression is a complex and dynamic phenomenon, which is not cell-autonomous, relying on the interactions and communication of the cancer cells with other cell types. Emerging data have highlighted the role of the tumour microenvironment, which interacts constantly with the tumour influencing its development: non- malignant cells of the tumour microenvironment (immune cells, fibroblasts, blood vessels) have a dynamic and often tumour-promoting function at all stages of carcinogenesis. Such factors, including hypoxia and metabolic disturbance, are known to be important determinants of brain cancer tumourigenesis. Hence, animal models represent the only viable model in which to undertake the glioma studies described here.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers have been estimated by taking into account:

1) The experimental plans, including the number and size of treatment and control groups required to generate meaningful results.

2) Our previous data performed in similar experiments over the last 10 years.

3) Data from published literature using animals in similar experiments.

4) Published data used to undertake calculations that inform animal number.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We designed our experiments to address a limited number of specific scientific questions that are based on a strong rationale and need, thereby reducing the number of required strains and breeding. We carried out calculations to make sure that the minimum number of animals is used to get a statistically relevant answer. To plan our experiments we also used guidelines such as PREPARE and on-line tools such as the NC3R Experimental Design Assistant.

An important step in designing our experiment is to control for or reduce any sources of unwanted variation, such as the ones deriving from age or sex of animals. We aim to use animals of both sexes in our experiments, and to balance the number of females and males in each experimental group. We also aim to use age matched mice in each group. All our experiments will be done by using mice on the same genetic background to avoid the influence of any possible genetic variation along the study. We also use randomisation and blinding to minimise experimental variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible littermates will be used as controls to minimise variability, maximise the use of the available animals, and reduce unnecessary additional breeding.

We will use established mouse strains which will avoid the breeding required for generating new transgenic lines.

We have applied our experience of previous mouse studies on IDH1 glioma models to inform the experimental design, thereby helping to reduce animal numbers.

At the end point of each experiment we will appropriately and accurately store all samples in order that they can made available for collaborators.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use genetically engineered mice carrying inducible (conditional) genetic variants that predispose to brain cancer. The conditional nature of the intervention limits the potential suffering to only the period following treatment with the inducing agent. Some of the models will then develop gliomagenesis (or possibly gliomas) over time, depending on their genotype. All the models used in the project require induction of genetic changes in adults, to allow for normal development. Induced mice will be compared with their control littermates, Any animals develop signs of potential glio such as weight loss, change in BCS or hyper act will be humanely killed To preserve and study brain tissue architecture some animals will be terminally anesthetised, perfused and fixed before applying a Schedule 1 method. To collect large non-haemolysed amounts of blood some animals will be terminally anesthetised and a large blood sample collected before applying a Schedule 1 method.

Why can’t you use animals that are less sentient?

Species that are less sentient than mice do not have the physiological similarity with humans or functional tumour microenvironment necessary to undertake the research proposed in this project. Furthermore, the longer life span of mice compared to less sentient species allows the time required for the multistep process of tumourigenesis. Since cancer onset and progression is dynamic, the use of animals that have been terminally anaesthetised is not possible. It is also not possible to use less sentient more immature mice for this study because faithfully modelling adult brain cancers requires the use of adult mice. Furthermore, the majority of the genes studied in the project are required for normal development precluding earlier interventions.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Since we propose to induce new mutants/new mutant combinations, we will perform pilot experiments in which animals will be monitored closely by the PIL holders and animal facility staff, to check the development of any adverse effect. Any information collected from these pilot experiments will be used for refinements that will be applied in the

following experiments. Whenever possible, the use of the most refined whilst scientifically justified routes of administration of recombinant agents and refined handling will be used. As animals age monitoring increases and recorded using bespoke scoring sheets.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will publish in accordance with the ARRIVE 2.0 guidelines to ensure data can be reproduced in other establishments. We will share date obtained at conferences and meetings.

All dosing according to LASA guidelines and we will make use of PRPEARE in advance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will check the 3Rs website (https://www.nc3rs.org.uk.), sign up for the NC3Rs newsletter follow seminars on the responsible research in practice website (https://www.responsibleresearchinpractice.co.uk/), and regularly discuss with colleagues. Finally we will also follow any advices regarding ways to implement the 3Rs given by NACWOs/NVS at our licenced establishment.

111. Creation and repair of ano-rectal fistulae in a porcine model

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Fistula, ano-rectum, therapy, stem cells, scaffold

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine what combination of stem cells and biomaterials has the greatest impact on promoting new tissue growth in an ano-rectal fistula track.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Currently there is no gold standard treatment for treating ano-rectal fistulae and achieving long term healing. Patients with active fistulae experience considerable pain and reduced quality of life.

The expected benefits for patients with ano-rectal fistulae include:

The potential for different therapies to be applied as less invasive surgical procedures. This approach would preserve the anatomy of the surrounding tissue and thereby prevent incontinence (inability to control bowel movement).

If successful, it may also be used for fistulae in other parts of the body (e.g. head and neck; this area is currently not under investigation).

What outputs do you think you will see at the end of this project?

One output from this study will be publishable data which will be disseminated through peer reviewed journals and national/ international scientific conferences/meetings.

An Additional output may be patentable product(s) (identified once detailed analysis of the competing market has been undertaken) which can be commercialised and offered clinically to patients (once regulated studies have been completed). If a successful product is identified, it is likely to take longer than 5 years (outside the duration of this licence) before being offered clinically.

Who or what will benefit from these outputs, and how?

Long term the additional benefits will be to the patients, approx. 2 per 10,000 of the population in the UK experience chronic and recurrent fistulae, a successful treatment outcome would significantly improve patient's quality of life, reduce hospital visits and in-patient time thus reducing NHS cost.

How will you look to maximise the outputs of this work?

Output will be maximised by presenting at clinical meetings to obtain relevant feedback in order to ensure the final approach can be clinically adopted explore collaborative opportunities both with regards to cell type and source, biomaterials and mode of application/delivery Present at relevant focused meetings/conferences

Species and numbers of animals expected to be used

- Pigs: 50

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The pig is an established model for ano-rectal fistulae. The model was developed and published by our group. The pigs anatomy, size, diet and physiology is similar to humans for this model and is the most appropriate translational model available. The model allows

fistulae to be created in pigs which mimic human ano-rectal fistulae and have similar pathophysiology. Smaller animals do not provide appropriate clinically translatable data.

Female pigs are used in preference to males as their pattern of glandular distribution (groups of specialised cells that produce secretion to aid lubrication) within the internal anal sphincter (muscles that help the anus to open and close) is similar to humans.

Typically, what will be done to an animal used in your project?

For each animal under the first general anaesthesia (GA), 3 ano rectal fistulae will be created using an indwelling seton (a surgical thread), a blood sample and any tissue biopsies may also be taken at this stage, (these will be used to harvest cells and used as part of the therapy). The animal will be allowed to recover.

Approx. 30 days later, under a second GA the fistula will be treated. The seton within the fistula track will be removed, the fistula curetted (i.e. cleaned out) to remove granulation tissue (old scar tissue). A different treatment will be applied to each fistula (based around a combination of different biomaterial and cells). An MRI/CT image may be taken and the animals allowed to recover. Additional MRI/CT images (under GA) may be taken at monthly intervals to assess the healing of the fistula track.

The duration of the treatment stage may vary between 2 -6 months depending upon the healing outcome under investigation. All Animals will be killed at the end of the treatment stage and tissue removed for histological and molecular analysis (i.e. the tissue will be analysed under the microscope and using different laboratory techniques)

What are the expected impacts and/or adverse effects for the animals during your project?

In our experience of this model, all animals tolerated the procedure well showing little discomfort over the duration of the study. Any pain was transient and was alleviated by pain relief following each surgical procedure. They did not experience any weight loss or abnormal tissue growth.

Animal suffering will be kept to a minimum by regular monitoring by experienced husbandry staff. Prior to, and following the creation of the fistula and treatment application, animals will placed on a softer mash diet for a approx. 3 days to ensure the production of soft stool and prevent straining. Pigs are normally fed a pelleted diet which is harder.

The use of immuno-suppressive drugs (helps to prevent rejection when cells from one individual are placed into a different individual) may be considered when it is not possible to obtain sufficient number of autologous cells (i.e. cells from the same animal) without impacting on the animal's physiological function.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of this model is 100% moderate.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project will address a clinical question i.e. is it possible to heal an ano-rectal fistula similar to that seen in humans using the different therapies proposed. As such, a clinically translatable (a treatment plan which is transferable to humans), relevant large animal model is needed. All work leading up to this stage that could be done either in vitro (in a petri dish or in the lab, specifically is the material cell friendly ie cytotxicity) or in smaller animals (potential material degradation and immune response) has been completed.

The next phase is to assess the combinational therapy (combined cells and biologically derived materials) for its intended clinical application and for this a large animal model is required.

Which non-animal alternatives did you consider for use in this project?

A non- animal alternative animal was not considered as we require a large animal model for direct clinical translation.

Why were they not suitable?

We require a large animal model for direct clinical translation and all necessary biocompatibility studies (i.e any toxicity relating to the materials, specifically immune response and material degradation) have already been conducted in rodents.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The experimental design is based on our previous studies (resulting in published data) using this model to assess a commercial collagen paste together with fibroblasts (special cells involved in healing). The previous data will also help to determine the group size, cell dosage, and the amount of biomaterial for each experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to minimise the number of animals, up to 3 fistulae will be created in each animal with no additional cumulative adverse effect thereby reducing the overall number of animals required. Control fistulae created will receive no treatment, however, based on previous experience/data, controls may be created per study and not per animal (i.e. it is not necessary for there to be a control in every animal).

Biological variability within the animal model will be controlled by using the same sex and similar weight animals and is based on our previously published study and experience.

The use of non-invasive assessment (e.g. MRI imaging), whilst potentially increasing the number of anaesthetics an individual animal has over the course of a study, can significantly increase the amount of information gained per animal and therefore reduce the overall number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Post-mortem tissue sharing between different projects and collaborators will also ensure maximum usage of each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The pig is the most appropriate animal model with regards to anatomy and physiology when compared to humans for the creation of fistulae of relevant size. In our experience of this experimental design, all animals tolerated the procedure well showing little discomfort over the duration of the study.

Ano-rectal fistulae created in pigs are unlikely to be as debilitating because unlike humans who sit on their “bottom” thereby placing pressure on the ano rectal fistula, pigs predominantly lie on their belly or on their side resulting in less pressure and soreness on the outer surface of the ano-rectal fistula

Additionally, this model allows for the creation of fistulas of comparable size and potentially similar complexity. Whilst this is the best model, we have been able to develop it is recognised that the pig is quadrupedal (stands on 4 legs) while man is bipedal (stands on 2 legs) resulting in different pressure distribution on the fistulae.

Animal suffering will be kept to a minimum by regular monitoring by the Named Animal Care and Welfare Officer (NACWO) and the Named Veterinary Surgeon (NVS) throughout the study. Prior to, and following the creation of the fistula and subsequent treatment, animals will be placed on a mash diet for a minimum of 3 days to ensure the production of soft stool and prevent straining.

All surgical procedures will be performed in line with human clinical practice, whilst the animal is under general anaesthesia (GA), (for animal welfare and practical purposes a GA is necessary). Pain relief will also be given where appropriate to ensure overall animal wellbeing.

Why can’t you use animals that are less sentient?

Less sentient species are not appropriate because the data generated cannot be clinically translated (i.e. studies of fistulae in rabbits or rats would need to be repeated as their physiology is very different).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

By acclimatising the animals to single housing prior to the initial surgery, any adverse effects or injury to the fistula site from pen mates can be minimised. Animals will be monitored regularly, pain relief administered (if appropriate) and given a mash diet for a minimum of 3 days to ensure the production of soft stool to prevent straining.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The use of best surgical practice adherence to the principles set out in the LASA (Laboratory Animal Science Association) guiding principles document combined with good pre- and intra- operative care and monitoring will minimise unnecessary suffering.

The Norecopa, NC3Rs and LASA (and similar animal research and welfare) websites

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will review the current literature (encompassing changes in veterinary research and human surgery) and any revisions to the regulatory guidelines along with input from the

local Named Information Officer (NIO),

Named Animal Care Welfare Officer (NACWO),

Named Veterinary Surgeon (NVS) and other local animal care staff.

As well as checking the Norecopa, NC3Rs and LASA (and similar animal research and welfare) websites and implement any changes where appropriate.

112. Tissue function and metabolism in heart disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

cardiac function, heart failure, metabolism, diabetes

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant
Rats	adult, juvenile, neonate, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To increase our understanding of the biochemical reactions leading to impaired energy supply in the failing heart, to limit worsening of heart function and to identify as well as test new drugs targeting metabolism.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Heart failure remains a major cause of death worldwide, despite great advances in medicine and therapy over the past few decades. Nevertheless, little is known about what makes hearts fail at the heart cell level. Thus, the key aim of the projects outlined here will be to advance our knowledge and understanding of the changes that occur in heart cells that cause hearts to fail. In turn, this information may help with early diagnosis and successful therapy for failing hearts aimed at improving energy supply in the heart cells by organelles called mitochondria (powerhouse of the cell). Where possible, information from animal studies (including diabetic heart disease) will be translated into studies undertaken by our collaborators who are clinicians involved in our studies. All the findings of the studies will be presented at conferences and published in academic journals (open access).

What outputs do you think you will see at the end of this project?

Experiments performed on this licence will help us understand how changes in cardiac metabolism lead to poor cardiac function and heart failure in order to develop new therapies and new ways to diagnose. All the findings will be presented at conferences, deposited in curated, public data repositories and published in academic journals. All project data will also be made available via research data management at our university.

Who or what will benefit from these outputs, and how?

Changes in the way heart produces energy by mitochondria (powerhouse of the cell) to power its work lead to series of changes to its structure and work causing it to fail. Our research aims to improve our understanding of the changes at the heart and mitochondrial level which underpin poor cardiac energy production by heart metabolism which triggers heart to fail.

Successful results of our studies, specifically identification of changes at the cell level and changes to energy producing pathways in diseased heart would also pave the way for new approach to treat failing hearts (including in type 2 diabetes) - something scientific research largely failed to achieve to date. They would be also be preventive as they would help us understand what leads to faulty mitochondrial causing energy starvation in failing heart as well as identify new and direct drugable target to treat heart disease (ie mitochondrial metabolic intermediates, metabolic transporters, immune system components).

Successful outcomes of our studies would also enable early diagnosis in clinics (blood sample analysis for metabolite and biochemical profiling).

Work generated using this licence would also identify novel concept with extensive and immediate translational potential for patients: for example re-purposing failed drugs for type 2 diabetes to treat faulty mitochondria and energy supply to stop and reverse heart failure in type 2 diabetes. Given that this drug has been fully approved for human use, any beneficial effect would be immediately translatable to clinical trials.

How will you look to maximise the outputs of this work?

Published (anonymised if applicable) data will be made available within 24 hours of the manuscript publication. Following consultation with the innovation office at our establishment, regarding any patentable IP position throughout the duration of the project, all data will be passed to the public domain in the form of open access publications and presentations at international conferences, once this is appropriate. All project data will also be made available via research data management group at our establishment. Published outputs will be assigned a DOI used to reference the data in publications and will be published under creative commons licence (CC-BY) allowing other to use, follow or continue the work. Appropriate metadata (Dublin core standard used; excel speadsheets with raw data, author names, study abstract) will be published with the research data to enable other researchers to identify whether the data could be suitable for their own research .Data will be managed, stored and curated by an open-access institutional repository of research outputs.

Species and numbers of animals expected to be used

- Mice: 2500
- Rats: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice and rats will be used for the studies as these are extensively validated animal models for study of cardiovascular disease including diseases of metabolism such as type 2 diabetes.To obtain relevant measurements of cardiac function it is necessary to use an animal model with a four chambered heart like the human heart. In addition, as metabolism and contraction of the heart are inextricably linked, it is essential to study metabolism in the intact beating heart.

Typically, what will be done to an animal used in your project?

We use both commercially available and bred genetic murinae models to investigate the role of specific gene changes in heart disease, obesity and diabetes. In most cases the animals do not show any overt adverse effects, however if any more severe phenotype may occur (such as hyperlgycaemia in diabetics) these animals will be monitored closely (tail blood sampling for glucose, insulin or markers of inflammation). In terms of dietary modification this may involve increased calories (western diet), introduction of metabolic modifiers to a diet such as glucose lowering agents, lipid metabolism inhibitors, vitamin supplements or metabolic sensitizers. On occasion, labile compounds such as vitamin B supplements or metabolic inhibitors may be given via oral gavage or injections. Non-

invasive imaging (echocardiography) will be used to non-invasively monitor heart function and reduce a number of animals required for the studies.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases the animals do not show any overt adverse effects, however if any more severe phenotype may occur (such as hyperglycaemia in diabetics) these animals will be monitored closely. Possible expected impacts may include weight gain, increased blood glucose, increased appetite and increased urination.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Total number of animals in the project: 5300

Number of mice:3900 (73.5%) moderate

Number of mice:1000 (18.9%) mild

Number of rats:400 (7.5%) moderate

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is commonly accepted by biomedical experts that animal models are required to fully understand the pathogenesis of complex human disease, as well as to test novel potential therapeutic strategies that research such as ours may unveil. The mouse models we plan to use are directly relevant to the human heart failure condition, a syndrome that accounts for a high level of deaths and for which there is currently significant unmet clinical need in terms of effective, life-saving therapies. To obtain clinically relevant measurements of heart function it is necessary to use an animal model with a four chambered human like heart. Furthermore, as metabolism and contraction of the heart are inextricably linked, it is essential to study metabolism in the intact contracting organ. Therefore, this study requires an integrated approach monitoring cardiac metabolism simultaneously with heart function and as such there is no appropriate alternative to using animals and isolated animal hearts

as the experimental model. Small animals, specifically mice, are widely used in the study of hypertrophy, diabetes, metabolic derangement and heart failure. We aim to use these validated approaches which provide hugely valuable information relevant to humans- which we have a lot of experience of using over the years.

Which non-animal alternatives did you consider for use in this project?

The HL1 cell line, a mouse atrial tumour cell line which represent many of the metabolic properties of the cardiomyocyte will be used where possible, for example to investigate the effect of changes in energy supply on function of mitochondria. Isolation of cells for primary cell culture offer potential for many studies including fluorescent studies but must still be collected from individual experimental animals models. The use of isolated perfused heart preparations has proved appropriate in our previous studies for investigating biochemical adaptations in parallel with analysing heart function.

Opportunities for studies on human heart tissue are limited owing to availability, sample size, and limitation in sensitivity of some of the methods used. We also employ computer-based prediction tools in addition to cell experiments to study targets. It is not currently possible to use solely computer models for these studies, although our findings may help guide future computer programs to help understanding of energy metabolism involved in heart failure.

Why were they not suitable?

Unfortunately, cell models of heart disease, heart failure and type 2 diabetes are limited in their ability to provide information that is useful in terms of translating experimental findings into therapy. Cell culture systems have complications of losing morphology, poor viability and are often derived from neonatal hearts rather than mature hearts. In addition, these do not provide an adequate measure of integrated heart function.

Human atrial samples are more likely to be available than ventricular tissue however they are less likely to be representative of heart failure. Specialised techniques for analysing changes at the cell or molecular level in disease require use of tissue samples as well as appropriate control samples - something that is not always feasible when using samples of human origin.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The minimum number of animals for statistical significance will be used, calculated using power calculations with information based on past experience and on consultation with biomedical statisticians.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Redundancy of studies will be avoided through good systemic reviews done prior to any study and the use of tools such as The Experimental Design Assistant (EDA) from the NC3Rs will help to ensure that the minimum number of animals will be used consistent with scientific objectives, methods to reduce subjective bias, and appropriate statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The minimum number of animals for statistical significance will be used, calculated using power calculations with information based on past experience and on consultation with biomedical statisticians. In the measurement of cardiac metabolism using energy substrates labeled with carbon 13 isotope in the isolated heart, we have designed experiments which determine use of two different substrates simultaneously, thus reducing the overall numbers of animals needed. Through regular exchange of information with other research groups in the University we ensure that, where possible, students requiring tissue other than heart coordinate their studies with those doing heart perfusion, thereby using one animal for two experiments

Longitudinal imaging (echocardiography) will impact on animal number; maximising experimental data obtained per animal through the acquisition of a large number of experimental outcomes (functional parameters).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Ultimately the critical issue involved in heart failure, is whether treatments lead to improved or deteriorating pump function. Thus, it is essential that integrated studies are carried out in which cardiac function is assessed in parallel or simultaneous with cellular or metabolic

manipulations. Creating experimental models of hypertrophy and administration of beneficial or deleterious compounds provides an in vivo model, which can then be studied in vivo, or at the organ, cell and molecular level by in vitro experimentation (as described above).

We measure cardiac function and metabolism in transgenic mouse models bearing a genetic modification identified in a clinically observed cardiac disorder or relevant signalling pathway. In some cases, we use a range of techniques to induce closely related disease phenotypes. These can give precise information about aspects of a disease or differences between stages of disease progression. The latter can also be modelled by allowing animals to age following induction of the phenotype, which may impose a greater stress on each animal than short term induction of a more severe phenotype but is more physiological and provides information on long-term changes resulting from the disease. In each case we select the most refined model to answer our questions Genetic models of diabetes will be used to explore specific changes to metabolic signalling. (ie type2 diabetic mice, obese mice, Zucker diabetic rats to study type 2 diabetes). When instituting a protocol that is new to our lab we perform a pilot study, after consultation of the literature and with collaborators, to confirm the appropriate protocol for the species and strain we are studying. There is the possibility of mortality in pilot studies, but we limit this as much as possible by careful monitoring of the animals for clinical signs of distress. We use these studies to define protocols and humane end-points to minimise mortality in larger studies.

We use non-invasive techniques wherever possible, eg dietary modification to elevate plasma fatty acids and giving therapeutic compounds in drinking water if applicable. For substances that need to be administered over a number of days, and cannot be introduced via the diet, we will use slow-release devices such as osmotic minipumps where possible.

Regular advice and updates on techniques, such as anaesthesia and surgery are sought from the NVS and NACWO, to ensure best practice. When implementing new procedures, where possible, arrangements to visit other departments where these techniques are already in place would be made. The animals are provided with environmental enrichment where possible, at the recommendation of the NACWO, including a variety of housing, tunnels and nesting materials.

Murinae (rats and mice) have been widely used for studies on the heart allowing functional measurements to be made relatively straight-forwardly and reproducibly and for providing sufficient tissue for further analyses. There is a large database of information about the rat and mouse hearts that ensures experimentation on models of disease can provide clear and definite outcomes.

Why can’t you use animals that are less sentient?

Alternative strategies to the use of animal models have been carefully considered but there are no non- sentient alternatives to answer many of the important medical, biochemical and physiological questions in the field of heart failure. A mouse atrial tumour

cell line, HL-1 cell line, does exist which has been shown to mimic many of the metabolic properties of the cardiomyocyte and this cell line will be used in place of animal tissue where possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The aim of this project is to use non-invasive techniques wherever possible and seek regular advice and updates on techniques, such as anaesthesia and surgery, to ensure best practice.

Discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research and analgesic, anaesthetic, and/or tranquillising drugs will continue to be used where appropriate to minimize pain and/or distress to animals

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Peer-reviewed, published literature will be a primary method for following best practice guidance as well as 3Rs advice from NC3Rs (including institutional 3Rs symposia, NCR3s page, NC3R events and workshops).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Peer-reviewed, published literature is a primary method for staying informed about advances in 3R. Redundancy of studies will be avoided through good systemic reviews done prior to any study and the use of tools such as The Experimental Design Assistant (EDA) from the NC3Rs will help to ensure that the minimum number of animals will be used consistent with scientific objectives, methods to reduce subjective bias, and appropriate statistical analysis.

113. Preclinical evaluation of cancer therapeutics

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Therapy, Immunotherapy, Pharmacodynamics, Efficacy

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project licence is to provide a high quality demand led service, testing the efficacy and tolerability of novel anti-cancer therapies in validated rodent models of cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This will provide data to support IND (Investigational New Drug) submissions for Sponsors, and assist earlier drug development strategies (go/ no-go decisions based upon pre-clinical efficacy).

What outputs do you think you will see at the end of this project?

There is an unmet need for new cancer therapies given relapse rates, the fact that the disease may become resistant to treatment and the percentage of patients that experiencing long-term treatment side effects. The aim of this project is to provide data from preclinical models of cancer to biotech and pharma in order to identify those compounds most likely to have the greatest clinical potential and highlight those with limited efficacy or serious side effects for rejection or reformulation as early in the development stage as possible.

In early stage drug discovery, therapeutic candidates are generally subjected to a series of in vitro screens to identify activity on a cellular level, without the need to use animal subjects. Testing the toxic potential of a drug on cells in vitro (screening against cell lines and primary cells from various tissues) provides some indication of potency and specificity compared to current therapies, but ultimately the most promising drugs need to be tested in an animal model that is as predictive as possible of the human condition. Animal models allow us to establish how a potential drug may be absorbed by the body and/or tumour, observe any beneficial effects and if/when they cease becoming beneficial and what interventions are needed. This means we can determine appropriate dosing routes and optimise the schedules with which treatment is given. We can then analyse the success of the intended drug- related effects on the disease. The most frequent and well characterised models are those in rodents, where one can demonstrate a degree of efficacy relative to a current standard of care drug. They also highlight those drugs that are either ineffective or have serious side effects (filtering out those potential drugs with little benefit or limiting side effects as early and as accurately as possible).

Who or what will benefit from these outputs, and how?

The initial benefits will be to the pharmaceutical industry. The predictive models we have developed and continue to refine provide efficacy and mechanism of action data that will enable informed decision making on both drug development selection/prioritisation and on optimal dose routes/scheduling in the clinic. By reducing the risk of later stage failures, there is cost/speed benefits for clinical trials. The benefit for them is therefore a reduced number of unproductive human volunteer studies (and a reduced risk of adverse effects due to dose scheduling errors) and most importantly the development of improved – more effective – cancer therapeutics.

How will you look to maximise the outputs of this work?

The information we provide to the industry is as extensive as we can provide, from in-life parameters showing efficacy of the drug, to analyses of tissues post-mortem that will help confirm the mechanism of action of the drug and perhaps identify biological molecules that may represent a response to therapy. With this data, we aim to support our clients to progress their drug into clinical trials and, in the longer term, be of benefit to patients as a standard of care. Because of the in-house experience and expertise we have with efficacy testing, it is conceivable that this will help minimise the number of animals that would have been needed, reduce time points and drug concentration variation but also acquire the most information out of an experiment without the need for repeats. Thus, our work certainly helps implement the 3Rs in practice.

Species and numbers of animals expected to be used

- Mice: 5650
- Rats: 1250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The programme requires that models are used which closely mirror the human disease situation. Adult rodents (rats and predominantly mice) are suitable for these studies as the work cannot be conducted in lower vertebrates, invertebrates or cell lines due to the poor resemblance of these options to the clinical setting.

Typically, what will be done to an animal used in your project?

Animals that have been obtained from a supplier will be acclimated to their new housing for at least 1 week. After this period, for tolerability studies, 1-2 animals will be dosed once or multiple times, using the same or increasing concentrations of test item. Careful monitoring of the animals will take place immediately after dosing to assess any dosing/drug-related effects, and periodically thereafter (typically twice a day).

Pharmacokinetic/pharmacodynamic (PK/PD) studies are used to determine how a drug is absorbed by the body, where it goes to and how long it stays there, and what effects it might have on the body. For PK/PD studies in non-tumour bearing and tumour bearing animals, small groups of mice or rats will be dosed with a drug (or the same solvent without any drug, if using controls) via a known route, and at a volume, concentration and schedule that is known to be well tolerated.

In cancer studies, animal models have been developed that show the disease at the relevant site, e.g., lung cancer models that develop cancer in the lung, as opposed to

subcutaneous models where the cancer cells are engrafted subcutaneously even if this is not where they would normally grow. These are described as orthotopic models.

For efficacy or PK/PD studies in animals with locally growing cancer, tumours will be established by subcutaneous injection of tumour cells. Careful monitoring of the animals and the implantation site will be performed, as well as normal health and well-being observations. Efficacy will be determined by achieving a desired effect on the disease with little to no unintended affects to health or well-being. This may include calliper measurements that can be performed on conscious animals with minimal distress. Test Item (or the same solvent without any drug, if using controls) will be administered via a known route, and at a volume, concentration and schedule that is known to be well tolerated.

For efficacy or PK/PD studies in orthotopic models, or animals with cancers that affect the entire body, tumours will be established by intravenous, intrafemoral, intraperitoneal (through the abdominal wall), intracranial, or intrathoracic injection of tumour cells. If the implantation technique requires aseptic surgical intervention, animals will be anaesthetised, and analgesia may be given during and after surgery. Careful monitoring of the animals and the implantation site will be performed, as well as normal health and well-being observations. This may include calliper measurements that can be performed on conscious animals and/or in vivo imaging of anaesthetised animals. Test Item (or the same solvent without any drug, if using controls) will be administered via a known route, and at a volume, concentration and schedule that is known to be well tolerated.

Blood samples may be collected periodically via a tail vein bleed in conscious animals. We will always follow the NC3Rs guidelines/limits for peripheral blood sampling.

Animals may be subject to in vivo imaging. This most often involves an intraperitoneal injection shortly before anaesthetising the animal for the imaging procedure. Animals will always be allowed to sufficiently recovery from anaesthesia (return to normal eating and drinking and peer interaction) before the next imaging session. Imaging is generally performed 1-2 times per week. For studies where we are trying to determine which organs/tissues are affected by the disease, which only a small proportion of animals will experience, optical imaging may need to be more frequent. The imaging sessions are about 5 minutes and as animals are anaesthetised with inhalable anaesthesia they recover quickly.

Recovery may be aided by keeping the animals warm and giving them mashed food.

Animals may receive radiotherapy, either locally or whole-body irradiation on one or several occasions, under anaesthesia. Animals will always be allowed to sufficiently recovery from anaesthesia (return to normal eating and drinking and peer interaction) before the next imaging session.

The endpoint for a study will usually be a specified timepoint, tumour size/burden (based on previous data and standard of care for the cell line) or if a humane endpoint has been

reached. At this time a final terminal bleed may be performed under terminal anaesthesia and tissues harvested after euthanasia for analysis. The length of the studies will vary, but generally falls between 1 and 3 months.

What are the expected impacts and/or adverse effects for the animals during your project?

Frequent adverse effects after test item administration may be weight loss and diarrhoea. Less frequent adverse effects may be bruising, anaemia, loss of appetite, reduced movement, grooming and postural changes. Infrequent adverse effects may be abnormal breathing, tremors, convulsions and reduced peer interaction.

Animals will experience stress due to restraint and transient discomfort from needle insertion and/or anaesthetic injection or inhalation of gaseous anaesthetics (100% incidence).

Repeated intravenous injection can result in irreversible damage to the vein and very occasionally, a haematoma/bruising may develop.

Intraperitoneal injection (through the abdominal wall) is likely to be painful if the needle injures an abdominal organ or if the substance being injected is an irritant.

Oral administration (gavage) is associated with minor discomfort. Very occasionally damage to the oesophagus may occur or substances may enter the lungs resulting in dyspnoea.

Slow-release pellets and osmotic minipumps are small and cause no appreciable effects. Animals may experience a lowered body temperature during anaesthesia. Animals may experience pain on recovery from anaesthesia.

Animals will experience stress due to restraint and a transient discomfort from blood collection (100% incidence). Animals may become overheated or dehydrated from spending too long in the hotbox.

Subcutaneous injections of tumour cells are associated with a momentary discomfort. Very occasionally, a haematoma or bruising may develop. Very rarely, tumours may grow to a size that could cause discomfort or interfere with animals ability to satisfy thirst or hunger. Rapidly growing tumours may ulcerate.

With all surgical procedures there is some risk of development of infections or wound complications (<1%). Use of genetically altered or immunocompromised animals may exacerbate the frequency of infections.

In cancer studies, animal models have been developed that show the disease at the relevant site, e.g., lung cancer models that develop cancer in the lung, as opposed to subcutaneous models where the cancer cells are engrafted subcutaneously even if this is not where they would normally grow. These are described as orthotopic models.

Orthotopic or metastatic tumour models may have various adverse effects, dependent on the site of implantation. Metastasis is clinically relevant, and there are a number of cancers that will initially grow at a primary site, but cells will break away, travel around the body and then develop as secondary (or metastatic) tumours elsewhere in the body. Common sites of metastatic disease after intravenous implantation include the lungs, liver, ovaries, kidneys and spleen.

For orthotopic models, such as leukaemia, animals may gradually become anaemic, weak, lethargic and lose body weight. Infiltration of the spleen or liver of disease can lead to enlargement of these organs which may be palpable.

For orthotopic/metastatic models implanted intraperitoneally, tumour formation may occur in one or several organs in the intestinal cavity. This may also lead to the formation of ascites (collection of fluid in the abdomen) which could cause discomfort, affect behaviour, and mask weight changes.

For orthotopic models in bone, animals may experience pain on recovery from anaesthesia and tumour formation may affect integrity of bone, invade surrounding muscle tissue, or hinder movement.

For orthotopic models in specific regions of the brain, animals may experience pain. Tumour formation may affect the physical condition and general behaviour, including displays of repetitive or uncontrolled movement, fitting, head tilting or abnormal responses to stimuli.

For orthotopic models of the thorax, tumour formation may cause discomfort and laboured breathing.

Animals receiving whole body irradiation, may experience a reduced appetite and hence weight loss and possible loss of condition. Radiation sickness evidence by diarrhoea could develop (less than 5%).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for mice and rats under all protocols under this licence is moderate. Based on the previous 5-year licence period, 85% of mice and 55% of rats are expected to experience the moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project requires that the models used are ones which closely mirror human disease. Animal models address issues which current in vitro tests cannot accurately determine such as the therapeutic index of a new agent, whole body pharmacokinetics (PK) and pharmacodynamics (PD). PK/PD studies are used to determine how a drug is absorbed by the body, where it goes to and how long it stays there, and what effects it might have on the body. Use of animals is needed to address the development and treatment of disease in an environment which is the closest to the microenvironment found clinically.

There is not currently an alternate representative of these systems that can be accurately studied that does not involve the use of an animal model.

Which non-animal alternatives did you consider for use in this project?

We have, and continue to, develop complex in vitro predictive models. We have validated 2D and 3D tumour culture models, including those that mimic the response of cancer stem cells vs a more hierarchical tumour tissue. However, to-date none are sufficient to replace the animal models themselves.

Why were they not suitable?

All compounds tested will previously have been screened using in vitro models to determine those candidates suitable for in vivo testing. These include studies that demonstrate the mechanism of action (eg. regulation of proliferation, induction of cell death, target cell specificity). Their limitation is, that although they allow assessment of test items on specific aspects of the disease process, they are insufficient in modelling the complex interaction of the different cellular systems present within an in vivo tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on the number of animals used during the previous 5-year licence period.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The most important aspect of the proposed programme of work, which will reduce the overall number of animals used, will be a careful selection of only those targets that offer a realistic prospect of therapeutic exploitation. This will be done on the basis of in vitro target validation studies and any available in vivo data, and work may be turned down if there are insufficient in vitro data available. In vivo models will not be used for random compound screening and all experiments will be hypothesis led. All experiments will be conducted following standard operating procedures and include appropriate controls, so that the efficacy of test items can be accurately assessed. The number of animals used will be the minimum number required to test the experimental hypothesis at the required level of statistical power and significance. We regularly consult academic and commercial statisticians. For example, data produced under the previous licence have been reviewed and the suggestions for improved analysis have been included in this licence application.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Several of our tumour models utilise imaging, which helps reduce animal numbers as the progression of disease can be followed in the same animal over time. We regularly seek statistical consultancy, sometimes providing historical data, to ensure that our power analyses are correct for each model we offer. Internal expertise in tissue analyses, allows us to fully characterise the cellular changes associated with disease as well as the expression of key factors on the surface of tumour cells and surrounding cells. This information is utilised to refine existing/validated models so that the most appropriate model is chosen for clients' compound's mechanism of action.

Outsourcing work using these models to CROs such as ourselves, results in a greater level of assay standardisation, reducing the number of studies performed. We are exploring the possibility of sharing control data between studies to further reduce animal use, where Sponsors will allow.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To test the efficacy of novel anti-cancer drugs it will be necessary to induce sufficient levels of disease. Some studies will be performed in immunocompromised mice, rats will only be used where suitable.

More severely immunocompromised mouse strains may be required for some tumour models, and when they allow for the testing of human based immunotherapies (in this case mice may be carrying a humanised immune system).

We will predominantly use subcutaneous cancer models in mice. Animals will receive treatment delivered by an appropriate route of administration. Treatment response is determined by 3x weekly calliper measurements of the tumour. These types of models are a good way to determine if a drug has anti-cancer activity, however they do not allow for all types of disease. For this purpose, we have established and validated imaging models of cancer using labelled tumour cells implanted into the normal anatomical position. Since the cells are labelled, tumour burden can be monitored in the same animal over time using imaging methods. The endpoint for several of these models is now based on imaging signal rather than humane endpoints, reducing suffering.

On occasion, syngeneic tumour models in mice may be used. These models are developed by administering tumour cells derived from the equivalent strain of animal, i.e., a mouse receiving cells derived from the same strain of mouse. They are most useful for sponsor's who wish to test therapeutics in a fully functional immune system. For subcutaneous tumours, some treatments may cause transient inflammation/ulceration, which has been shown to resolve, and the mice then produce useful data with minimal residual distress from the skin. Hence, a transient window is needed to prevent premature termination due to a short-term effects. Ulceration is a clinical sign of a well-functioning immune response. However, tumours will be monitored and scored 3x weekly, and animals with increasingly significant damage, (tumour score = ulcerated), will be humanely euthanised by a schedule 1 method.

The frequency will depend upon the cell type and the kinetics of tumour growth. Where possible, cell lines that induce ulceration will be avoided. This is particularly relevant in therapies targeting the immune response to the tumour which may necessitate the allowance for ulceration.

Why can't you use animals that are less sentient?

Rodent models are the best characterised of all experimental oncology models, with mice being much more heavily investigated than rats. Furthermore, work cannot be conducted in lower vertebrates, invertebrates or cell lines due to the poor resemblance of these options to the clinical setting. Rodents provide a cost and time effective platform in general for most pre-clinical testing. For the purposes of oncology testing, the use of higher species is not required because there is a wealth of knowledge on different types of cancer in rodents, as well as decades of in-house expertise with such models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over the lifetime of our previous licence, we have established and validated several imaging models, where the disease burden can be monitored in the same animal over

time, which means that less animals are used. The endpoint for several of these models is now based on imaging signal rather than humane endpoints, which means less suffering. Furthermore, we have refined the tolerability protocol and lowered the severity from severe to moderate for this protocol in this licence application. We have increased the number of different data read-outs that can be obtained from a single study.

We have introduced an ulceration score sheet to refine our tumour observation methods. This will help to ensure all staff are aware of what constitutes an 'ulcerated' tumour, and the endpoints are consistent. It will include descriptions of the appearance of the tumour; timelines based on historical data, i.e. time taken from implantation of cells to ulceration of tumour; and visual aids that can be updated for different cell lines and mouse strains. The frequency of monitoring will alter as the appearance of the tumour site changes, i.e. the frequency will increase with the severity of the observation.

We will continue to refine our models and anaesthesia and analgesia will be employed for any surgical procedures. Blood sample volumes and number of samples will be kept to a minimum.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Workman P et al. (2010). Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102(11):1555-1577.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are regularly getting informative emails from the NC3Rs and we make sure to attend seminars and workshops about the subject. We are keeping up to date with the literature within the field of pre-clinical cancer research and are actively looking to implement refinements of our models. Our standard operating procedures are reviewed on a regular basis to accommodate refinements.

114. New therapeutic strategies to treat chronic pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Chronic pain, Therapy, Sensory Nervous System, Neurophysiology, Analgesia

Animal types	Life stages
Mice	adult
Rats	adult
Guinea pigs	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our aim is to expand knowledge and understanding of the mechanisms underlying the development and manifestation of chronic pain states and to use this knowledge to develop new therapeutics and novel strategies to treat these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic pain has been described as "The Silent Epidemic". It is estimated to affect more than 40% of the UK population reporting pain that persists 3 months or longer. Incidence increases with age; prevalence among over 75's may be as high as 62%. Chronic pain is associated with high incidences of anxiety, depression, sleep disturbance, insomnia and decreased physical activity. There are also broader implications, with chronic pain sufferers less likely to be in full-time employment. A range of pharmaceutical strategies have been used to treat patients but these are frequently ineffective, especially in severe forms of pain. A consequence of this is the ever increasing use of opioid-based drugs, the chronic use of which is associated with severe side effects including, respiratory depression, sedation, tolerance and dependence. The social consequences have been devastating with an increasing rate of prescription opioid overdose deaths occurring across developed societies, "The Opioid Epidemic", with an estimated 90,000 casualties estimated in the US alone in 2020. The socio-economic burden is similarly highly significant, the annual cost of chronic pain in USA estimated around $600 billion (i.e., expenses, lost income, lost productivity). The cost of medication to treat adult patients with chronic pain in the US is estimated >$17 billion annually and forecast to increase. The numbers are staggering, the treatment of pain represents a significant current and future medical challenge owing to the shortcomings of current analgesics together with the largely unmet clinical need is beyond question.

The work outlined in this project will directly contribute new knowledge of the fundamental mechanisms contributing to the development and manifestation of chronic pain states and provide intelligence to drive new therapeutic strategies without the adverse side-effects associated with current treatments.

What outputs do you think you will see at the end of this project?

We will produce data that significantly advances our understanding of the mechanisms underlying chronic pain, in particular pain associated with nerve damage.

We will produce peer reviewed publications and present data at scientific conferences.

We will produce data sets to be shared with academics and the biotechnology/pharmaceutical industry.

We plan to produce data and intellectual property that can be patented.

We plan to produce data that informs clinical development of new treatments for pain and new therapeutic approaches to treat pain.

Who or what will benefit from these outputs, and how?

In the short-term, research papers and presentations at national and international conferences will be of benefit to both research scientists in academia and clinicians. This work will also be of interest to industry including pharmaceutical, biotechnology and medical device sectors. The new information will enhance our understanding of mechanisms contributing to chronic pain at the single cell, tissue and whole organism level.

In the short to medium term, the biotechnology and pharmaceutical industries will be interested in new therapeutic targets to treat chronic pain states, and in new therapeutic strategies to treat these conditions through alternative approaches such as medical devices.

In the medium term, the development of intellectual property, patents, investment and new companies will facilitate creation of new jobs and economic benefits. With our significant assets already in the pipeline, not yet patented, we are ready to move forward to early stage clinical development over the course of this license.

In the longer term, 5 years and beyond the term of this license proposal, the beneficiaries will be patients suffering chronic pain. Delivering successful pain relief without the adverse side-effects associated with current medications will deliver significant health and socio-economic benefits.

Other beneficiaries will be pain clinicians, all of whom complain and highlight their current "lack of tools" to treat chronic pain.

Finally, animals are likely to benefit as treatments developed for conditions such as pain and epilepsy are frequently used to treat domestic animals.

How will you look to maximise the outputs of this work?

We maximise outputs from this work by disseminating new knowledge through research publications and presentations at conferences.

We also actively engage with a network of industry partner collaborators globally where key data on "gold-standard", or positive control compounds are made freely available and shared, improving data quality, reliability and reducing the need for animal use to repeat experiments unnecessarily.

Scientific reports are shared with permission of collaborators with other industry collaborators providing key intelligence on pain targets whilst not disclosing compound details.

Our extensive experience over the past 20 years or more and going forward puts us ideally placed to advise on the highest quality, efficient preclinical development strategies for industry collaborators and partners, ensuring minimal requirement for animal use and suffering.

Historical data from our lab will always be used to advise industry collaborators on the validity of their therapeutic targets and likelihood of success or failure of their approach.

Species and numbers of animals expected to be used

- Mice: 4000
- Rats: 7500

- Guinea pigs: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will principally use mature (6 weeks and older), male rats and mice to investigate mechanisms underlying changes in nervous system function in chronic pain. Adult rodents are required as they have a mature fully-developed nervous system, unlike neonatal animals that are still growing and undergoing development, rendering it impossible to separate changes in nervous system function, resulting from chronic pain from those associated with development. Although we will primarily use males, as females have different pain responses, and differ in pain development during different stages of their oestrus cycle, we will also use female rats and mice in these studies. As guinea pigs have certain attributes more comparable to man, we may on the rare occasion that this is the case, use these animals.

Typically, what will be done to an animal used in your project?

Our aim is to generate model animals that mimic human pain/hypersensitivity scenarios and to achieve this we use procedures, outlined below, that cause nerve injury or inflammation and sensitization. The vast majority of models developed are associated with minimal discomfort but show hypersensitivity to an external stimulus such as touch or heat which is carefully monitored for all models. A range of techniques are used to investigate behavioural responses and electrical activity in the nervous system, and at the conclusion of the experiment, tissue may be harvested for further analysis.

Prior to performing any procedures, we test each animal for abnormal hypersensitivity to touch or heat. This is done by applying a light touch test stimulus (von Frey filaments), or a focused heat stimulus, applied directly to the paw. Those that do not have a normal sensitivity to sensory stimuli are excluded from further studies (approximately 2-5%).

Surgical procedures to generate animal models: For nerve or tissue injury models, animals are anaesthetised and either a nerve, muscle, tendon or bone is manipulated. Following complete recovery of consciousness and freedom of movement, animals are returned to their home cages. These procedures take approximately 10 minutes to perform per animal.

Procedures involving injection of substances to generate animal models: These include models of cancer chemotherapy, where animals undergo tail vein injections twice a week (maximum of 5 injections) with chemotherapeutic drugs. A fibromyalgia model where reserpine is injected daily for 3 days (under the skin or in the muscle). Inflammatory pain models where the agent is injected into the foot (once only) or knee-joint (two injections over two days). A Parkinson’s disease (PD) model where the chemical is injected daily for

4 days, or under anaesthesia, two small holes are drilled in the skull and a chemical injected into precise areas of the brain at a single dose'. In this case, the holes are secured with dental glue, and the animal allowed to recover in a temperature-controlled chamber before returning to the home-cage and their cage mates.

Administration of drugs in animal models: Potential new medicines will be administered during studies of animal behaviour, and during experiments to monitor changes in electrical activity of nerves. Drugs may be administered orally in food or water, or via a flexible tube inserted into the stomach. Chemicals may also be administered by injection delivered into the fluid surrounding the spinal cord or directly into the brain via thin tubes that have been implanted under anaesthesia prior to experiments with test chemicals being undertaken. In some instances, injections may be involve directly puncturing the skin with a needle and guiding the needle to the target without the need for surgery.

Behavioural tests: Animals that have undergone procedures described above will be assessed using behavioural techniques. Animals are placed in specialized chambers and allowed to acclimatise to this environment. Sensitivity to heat is tested using infra-red illumination and sensitivity to touch is tested using a brush or fine plastic fibres of different sizes, (force; von Frey hairs) applied to the hind-paw or cheek until they slightly bend, very much like the way these hairs are used in the clinic. The intensity of stimulus is gradually increased until a withdrawal response is observed indicating discomfort. Weight bearing tests will determine whether one hind leg is favoured indicating discomfort in a limb; grip force test will be used to measure changes in paw strength; movement and coordination will be tested using an accelerating rotarod and narrow beam walking tests. All behavioural tests are repeated to ensure data accuracy and animals are rested between repeated tests. Each test session takes less than 5 minutes. The maximum number of sensory stimuli any animal will be subjected to is 42 in any one session, with a maximum of 4 sessions in a day spaced at least 1 hour apart with animals returned to their home cages to rest between sessions. In long-term studies, these behavioural tests may be repeated once a week for between 2 and 24 weeks. For movement and coordination tests, animals are trained on the devices prior to testing.

Labelling of nerves for recordings: In some animals, we will record electrical activity from specific nerves to investigate hypersensitivity signaling. Nerves will be identified by labelling with a dye/tracer or virus expressing fluorescent proteins, injected into the nervous system (brain or spinal cord) under anaesthesia. This recovery surgery takes no longer than 30 minutes. Following recovery from surgery, animals are returned to their home cage for 2 to 5 days for dye injections, or housed for between 2 weeks and 8 months for virus injections. Animals are then humanely killed or placed under terminal, non-recovery anaesthesia and tissue is used for nerve-recordings of electrical activity.

Monitoring blood pressure, heart rate and electrical activity in nerves: Recordings of electrical activity in the nervous system, blood pressure and body temperature will be undertaken in freely moving animals using either wire-free or wired-connected devices. Animals will be placed under general anaesthesia and sterile devices implanted into blood

vessels, on the surface of the brain or attached to fine needles positioned in discrete regions of the brain. Following recovery from surgery, these devices allow blood pressure and brain activity to be monitored in the awake moving animal. For devices connected by wires, sterile, head mounts will be implanted under the skin and secured with dental cement to the skull. Following this procedure, animals are housed individually for at least 7 days before being returned to their home cages and cage mates. On the day of the experiment, animals are transferred to soundproof recording chambers where continuous monitoring of brain activity, blood pressure and core body temperature is undertaken over a 3-hour period.

Monitoring electrical activity in nerves under terminal anaesthesia: Recordings of electrical activity in the nervous system will also be undertaken in animals under anaesthesia. In these experiments, animals will be put on a ventilator and anaesthetic or test compounds will be administered intravenously through implanted tubing. Body temperature is monitored and maintained with a temperature-controlled blanket. Samples of blood (no more than 4 samples over 2 hours) and brain fluid (no more than 6 samples taken over 2 hours) may be taken to monitor the animal throughout the experiment. Nerve recordings are conducted through implanted electrodes placed in discrete regions of the brain or spinal cord. Recording will be performed over a period of between 1 and 6 hours. At the end of the experiment, animals will be humanely killed using an overdose of anaesthetic.

Number of procedures: Throughout these studies, no animal will be exposed to more than 4 procedures including a maximum of 2 surgical procedures. Animals typically experience one surgical/chemical procedure, or virus/tracer injection. Animals are closely monitored in the hours and days following surgery. Animals typically undergo one type of behavioural test, in some cases with repeat trials.

What are the expected impacts and/or adverse effects for the animals during your project?

The vast majority of animals in these studies will not show any indication of ill health or significant loss of quality of life. Animals in all studies continue to interact socially and are not expected to lose weight. All animals will be monitored and any animal that is likely to exceed the severity limit will be humanely killed.

Animals that undergo recovery anaesthesia with minor surgery to generate models of pain are expected to recover completely within 24 hours and behave normally. These animals and those in which chemical injections are used to generate models of pain, will be more sensitive to heat and/or touch in one limb or area for the duration of experiments, ranging between 3 to 5 days, to 2 to 4 weeks in the majority of cases. They often show a change in gait and may have spontaneous foot lifts of the affected paw or may protect the affected area. Most experiments will be complete within 4 weeks, but on rare occasions these studies will run up to 6 months, these latter studies only undertaken in a small proportion of animals when the effects of repeat dosing with a potential new medicine or treatment are tested. The condition and welfare of all models is carefully monitored throughout and

on the rare occasion that an animal is identified likely to exceed the severity limit, it will be killed by anaesthetic overdose.

Parkinson’s disease animal models may show abnormal behaviour, specifically they have difficulty maintaining balance and co-ordination when moving. This behaviour may last for several weeks.

Some animals will have minor surgery or an injection into the body or a limb to induce a short-lasting state of hypersensitivity but will recover rapidly from these interventions. The discomfort is typically

transient in nature with only a minority showing any sign of discomfort, for example a slight limp or lack of use of the affected limb. These features are usually lost after 6 to 48 hours and animals behave normally, interacting socially, being mobile and easily able to self-care. In the case of a facial nerve, increased facial grooming for the first 2 to 5 days following surgery can be seen but this again diminishes after 5 days and the animals behave normally. As with all models, these welfare and condition of the animals are closely observed and monitored. Animals that have devices or catheters implanted are expected to recover rapidly within 1 day post-surgery and will be given pain killers and post-operative care whenever necessary.

Some animals will be used under terminal anaesthesia for nerve recordings. However no adverse effects are anticipated and animals are humanely killed using an overdose of anaesthetic on completion of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rats and mice with surgically induced chronic pain: Mild (10%) to moderate (90%)

Rats and mice with chemically induced chronic pain: Moderate (90%) with a minority classified as severe (10%). Those classified as severe will recover well within 1 to 2 days.

Rats and mice with symptoms of chronic pain induced by a physical intervention, for example models with UV light-induced blisters, will have a severity rating of moderate (75%) or mild (25%).

Freely moving rats or mice with implanted devices or tubes will have a moderate (100%) severity rating.

Rats or mice injected with dyes or viruses: Mild (50%) to moderate (50%) severity rating.

What will happen to animals at the end of this project?

Humanely killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The research questions we seek to address requires the use of animal models. Pain is complex, arising from integration of activity within a fully functioning nervous system that can only be investigated in models with intact neural circuitry comparable to that involved in human pain perception. Thus, no suitable alternatives exist to address underlying mechanisms contributing to pathophysiology associated with chronic pain.

Experiments carried out on in vivo animal models will be limited to those questions that be answered using other, alternative methods. cannot

Which non-animal alternatives did you consider for use in this project?

Over the course of our previous work, we have introduced multiple in vitro assays into our research wherever possible. These include human cell lines, cultured neurones, dissociated neurones, ex vivo isolated nerve preparations and ex vivo brain and spinal cord slice preparations. Once molecular targets/pathways are identified, detailed mechanistic studies will use in vitro systems. By concentrating efforts on the isolated tissue/cell systems for detailed studies on mechanism of action of new therapeutics, we anticipate being able to reduce the number of animals used over the course of the studies.

Data generated over the course of this project is subject to mathematical/systems biology approaches, seeking to develop predictive, computer-based models of neural networks in chronic pain states. Two models are under development: one defines abnormal electrical activity in peripheral nerves associated with chronic pain. The second model details the functional operation of neural circuits in the spinal cord in chronic neuropathic pain. Details of the data underpinning the models and the models themselves will be made freely available for use in future experimental design and to reduce the number of animals needed in research.

Recording abnormal electrical activity in animal models of chronic pain is technically challenging and takes years to train and develop the knowledge to maximise the potential of experiments. Currently the only way to develop these skills is by gaining “hands-on” experience with live samples. We have engaged a company to design virtual reality training programs. By creating a virtual reality lab environment the aim is to train students and staff in both the practical and theoretical aspects of recording electrical activity in nerves and through running simulations of how to maximise the quality and potential of

experiments. We anticipate having this training aid available over the course of this new project and will be made widely available as a training tool. We believe these tools will significantly reduce the need for animal models and tissue samples.

Why were they not suitable?

Ultimately chronic pain syndromes manifest via interactions of multiple interconnected neural circuits spanning the periphery, spinal cord and brain and cells and tissues of the body. This level of complexity cannot be fully replicated in in vitro systems. Computer models are in their infancy and seek to replicate aspects of these systems and considerably more data is required to provide the intelligence needed to inform the models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates outlined here are based upon studies undertaken during our previous projects. We have over 20 years of extensive experience using these models and are therefore ideally placed to accurately gauge the group sizes required to deliver statistically meaningful data. Group size is continually assessed in a data-driven manner to identify every opportunity to reduce group sizes wherever possible. The most up-to-date statistical methods will be used to inform data analysis and experimental design, again to ensure sample size is driven to a minimum.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We make use of all support and resources available to us. We engage our NACWO regularly and our consultant statistician at all stages of data analysis and experiment design. Other resources that we use are the NC3R's Experimental Design Assistant, the ARRIVE guidelines and the Norecopa PREPARE guidelines. We also use FELASA guidelines for a number of reasons but also to inform our experimental design.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have made and continue to make considerable advances in our use of isolated cell culture and tissue systems in the experimental design. Our computer models of neural circuits are sufficiently advanced to guide experimental design in some instances and will lead to reduced animal numbers over the course of these studies. By implementing these approaches in the early stages of the experimental design, to provide key intelligence to inform experiments in live organisms, we anticipate significant reductions in the numbers of animals required for live animal studies as these projects progress. Based upon our extensive experience and significant databases we have joined an initiative with industry colleagues to share data generated with "gold- standard" compounds i.e., compounds used in the clinic that act as a positive control in studies. This initiative is designed to reduce the numbers of animals used in research by reducing the need for some control groups.

We will also ensure animal numbers are minimized by analysing tissues in multiple ways and wherever possible we will supply tissues to other researchers and vice-versa. Wherever necessary, pilot studies will be undertaken to optimise experiments and their design before committing to a full study. In most models we are able to use the side opposite to the site of injury, reducing the need for separate control models, thus reducing animal numbers. For studies with genetically modified organisms, we will acquire the models from external suppliers to avoid duplicating breeding programs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a range of models that mimic clinical symptoms with each model representing a chronic pain condition with different underlying aetiology and pathophysiology. Since no model encompasses all symptoms and elements of chronic pain, we use multiple models in order to capture scenarios as close to clinical reality as possible. The rat and mouse models outlined here are used as they are the lowest vertebrate group where well characterised, minimal severity hypersensitivity models of pain have been developed and characterized. All of the models outlined either target a specific part of the nervous system or a limb thereby restricting symptoms to localised areas whilst reducing suffering, distress or lasting harm to the animals. Other models directly mimic a human condition and are controlled to minimise animal suffering. Furthermore, the preclinical models outlined here

are extensively studied, documented and exhibit the classic behaviours associated with acute and chronic pain, including elevated sensitivity to mechanical and thermal stimuli.

Nerve damage models of pain are used where the nerve of a back leg of an animal is ligated (tied up) with the exception of a model of trigeminal neuralgia where a facial nerve is ligated. These models are prepared surgically under anaesthesia to minimise suffering and animals recover quickly. The pain in these models is localised, again minimising the harm and discomfort to the animal as a whole. Other models are used that directly mimic human conditions in which we control the treatment to mimic the condition but cause the least pain, distress, suffering or lasting harm to the animal. Chemotherapy- induced neuropathic pain is also modelled in-line with the clinical scenario where animals are treated with chemotherapeutic agents such as paclitaxel, resulting in peripheral neuropathic pain, just as occurs in the clinic. There is no alternative to this approach that would mimic the clinical scenario.

Similarly, myalgia (muscle pain) is modelled by injection of the chemical reserpine into muscle or subcutaneously. Parkinson’s disease (PD) and PD-related pain is also modelled chemically, inducing selective loss of dopamine-producing cells in the brain to mimic the same loss in patients. Surgical or post-operative pain is induced by making a surgical incision on the surface of a hind limb under anaesthesia, mimicking the human condition, and sensitivity to touch or heat stimuli can be tested as early as 3 hours and up to 3-4 days post-induction. Thereafter the animals recover rapidly thus minimising the distress and suffering to the animal. Bone fracture pain is modelled by gentle fracturing of the tibia in a rear limb, under anaesthesia, the affected leg bone being secured using a steel pin (all as part of the same procedure), again to minimise the discomfort and suffering of the animal.

Inflammation is induced by injection of chemical irritants into a hind paw or knee joint capsule with the exception of the blister model in which a concentrated UVB light source is used to create a blister on a rear paw. The models are all minimal hypersensitivity models designed to cause minimal distress and suffering to the animal whilst featuring hypersensitivity to external stimuli, hypersensitivity to touch and temperature being principle outputs that we monitor. In all of these models the chemical irritant is administered to a highly localised, restricted area and the animals rapidly recover within a few days, thus minimising animal suffering and harm. Hypersensitivity to touch is tested with fine fibres of increasing force, von Frey hairs, the force of fibres needed for the model animal to withdraw the limb being recorded, similar to the clinical scenario. Similarly, for hypersensitivity to heat, a paw is subject to increasing temperature and withdrawal of the limb from the heat source monitored. Animals are only exposed to the minimum mechanical or thermal stimulation level that they find uncomfortable and freely withdraw from, thus minimising any suffering, distress or lasting harm. Studies of abnormal electrical activity and the effects of new medicines are for the main part undertaken under terminal anaesthesia, eliminating any pain or distress to the animal. A series of studies recording electrical activity in freely moving animals is achieved by surgically implanting wires and

probes under anaesthesia to limit harm and discomfort to the animal prior to testing the effects of new medicines on brain activity, blood pressure and body temperature.

All of the animal models and scientific techniques outlined here were designed to mimic and record clinical conditions and do so with the minimal, harm, distress or suffering to the animals. They are the most scientifically extensively studied, validated and reported models and are sensitive to known “gold- standard” analgesics used clinically. We will only use those models which are most scientifically relevant to the questions posed and will use the minimal number of procedures to address each question to ensure animals are exposed to the least discomfort, distress, suffering or persistent harm.

Why can’t you use animals that are less sentient?

Our aim is to understand and recognise mechanisms underlying chronic pain and to identify new therapeutic approaches and treatments. Chronic pain syndromes are complex phenomena with multiple causes associated with extensive changes in peripheral nerves, in the spinal cord and brain leading to alterations in behaviour. They can therefore only be investigated in mammalian models with the lowest sensitivity and yet an intact, readily identifiable neural circuitry that is comparable to that involved in human pain perception. The rodent brain is by far the best characterised model for this purpose.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all animal models, animal health and welfare is carefully monitored and documented using established criteria (appended with license), based on adverse effects (exceeding moderate levels on the FELASA working group recommendations, 1994). Biomedical Services Unit staff and our own staff carefully monitor and report any signs of abnormal behaviour or animal health and welfare issues. For any experiments that require behavioural tests, animals are first trained and acclimatized to the environment for testing and all animals are housed in accordance with the home office guidance to maximize their environmental welfare through enrichment. All surgeries and whole organism nerve recordings are carried out under anaesthesia by highly trained staff. Experiments that require implantation of drug delivery devices or recording devices are also performed under anaesthesia with post-operative pain relief to maximize animal welfare and minimize harm. Analgesia is administered wherever possible following surgical procedures. We will continually interact with our academic collaborators, pharmaceutical/biotechnology partners, modelers, statisticians, veterinary surgeon and biomedical services staff will ensure refinement of existing and novel approaches.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Resources that we use are the NC3R’s Experimental Design Assistant and the Norecopa PREPARE guidelines. We also use FELASA guidelines for a number of reasons but also to inform our experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will meet frequently and communicate efficiently with the NACWO, Named Veterinary Surgeon (NVS) and staff at our BSU. Our NACWO keeps us informed of developments with the NC3Rs, relevant scientific papers and organises seminars specifically on the NC3Rs. We will attend NC3Rs webinars and conferences wherever possible.

115. Pathways of filopodial formation

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cell motility, filopodia, cell biology, actin cytoskeleton

Animal types	Life stages
Xenopus laevis	adult, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand how the cells make finger-like projections, called filopodia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Filopodia are produced when cells move, and they are thought to be sensory, in helping cells find the directions they need to go or how to make contacts with other cells. As a fundamental structure of cells they are widely important for health and disease, in the brain, in cancer and infection as three examples. In the brain, filopodia are produced when nerve cells make connections with each other, which is important for learning, behaviour and mental health. 3-4% of children in the UK are affected by neurodevelopmental disorders, and more than half of those referred to child and adolescent mental health services are found to have a neurodevelopmental disorder (such as autism). A second

example is the spread of cancer cells outside the original organ that they came from, called metastasis. Cancer causes 28% of UK deaths and metastasis is estimated to be the cause of 90% of them. Metastatic cancer cells have many filopodia and we do not understand what these filopodia are doing or how to change them. Filopodia are also formed when viruses infect cells, e.g. human immunodeficiency virus and the COVID virus where they are assumed to spread viral infection around the body. If we can understand and control how cells make or turn off filopodia we might be able to come up with new therapies in the future in any of these areas or others.

What outputs do you think you will see at the end of this project?

We will discover new information about filopodia. We will understand more about where filopodia form, how they reach certain lengths, how long they last for, and how they are suppressed.

The main output of the information will be publications reporting our findings. We will also present our findings at local, national and international scientific meetings. We will generate data that supports future funding applications. There may be new intellectual property that supports a patent application and we may generate data that supports clinical trials in the future.

Who or what will benefit from these outputs, and how?

In the short to medium term, other researchers studying filopodia and related aspects of cell biology will benefit from the outputs by being able to make new discoveries in their own work using the information we found out. In the long term the information we obtain may be useful information for people working on disease and in the very long term may benefit patients. Our data may lead to new ideas for therapies and the scientific tools that we create may be the foundation for new approaches to control filopodia (such as antiviral medicines or anti-cancer drugs).

How will you look to maximise the outputs of this work?

We will disseminate our work in conferences and collaborate with other researchers. We will also disseminate our work by making short videos for a lay audience and present our work at events such as festivals for the general public. We will make the computer code that we use publicly available and upload our manuscripts to a preprint server. Our work will be published open access so anyone can read it for free. Where our work is directly applicable to disease we will engage with patient charities. We will consider publishing unsuccessful approaches too, for example in supplementary information of other papers or as preprints.

Species and numbers of animals expected to be used

- Xenopus laevis: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using adult female African Clawed Frogs and their tadpole stages before they are independently feeding. We are using adult females to obtain their eggs. We use the tadpole stages where nerve cells are moving from the eyes to the brain of the tadpole as this is a well-understood system where filopodia are important and is a native biological system with immature animals.

Typically, what will be done to an animal used in your project?

The female adult frogs are injected with a hormone produced by horses during pregnancy. The frogs are injected under the skin at the bottom of their backs, just above their legs, into a place that contains a sac of fluid, which helps the hormone spread through the body. The hormone causes the egg cells to mature into eggs, mimicking what would happen to the frogs when they produce their eggs normally (e.g. in the spring). The immature egg cells finish their growing process, become eggs and are then able to be fertilized by sperm. A second injection 3-10 days later, of a hormone produced during human pregnancy and purified from urine (the hormone which is detected by pregnancy tests), makes the frogs lay the eggs. The differences between the horse and human hormones have the different effects in maturing the eggs or inducing laying.

After they are injected the frogs are put back into tanks of fresh water. After 5-15 hours the frogs are placed in a specific salt water solution for no more than 20 hours that keeps the eggs fresh once they are laid so that we can use them for several hours after laying. The salt water solution keeps the conditions outside similar to inside the frog's body. When the frogs have finished laying they are put back into fresh water. The frogs undergo the procedure more that 3 months apart. Some frogs lay better eggs than others, if a frog lays bad eggs on more than one occasion and needs to be killed, we will do so humanely.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected beyond transient discomfort of injection. Very rarely, in 0.1% of superovulation procedures, the frogs do not properly lay their eggs and develop a hard, bloated abdomen and become lethargic (become egg bound). This can be a delayed response up to a week after the procedure. Rarely there are mild stress responses that may be related to vibrations or the salt water such as sloughing of their skin in larger patches.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Xenopus Mild 100%

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We use animals to make extracts from the frog eggs and to understand filopodia during tadpole development.

Filopodia are small cellular projections which come and go very fast and use similar building blocks as other processes going on inside cells. This makes filopodia hard to study as we cannot see them for long enough and it is difficult distinguish which effect comes from which process in the cell. Extracts from frog eggs can be used to make structures similar to filopodia outside cells which are more stable and not confused by other processes. We use frog egg extracts because they allow us to attain very high levels of sophisticated molecular information, like which types of the building blocks are needed and which ones are used together.

To convert our insights using extracts back to real filopodia we need to use cells. We use cell lines for some of our work however the filopodia produced by cell lines are not fulfilling a proper biological function so we don't know if what we are seeing is meaningful. The nerve cells in frog tadpoles are a well-understood model that use filopodia for finding their way and connecting to other cells. We use tadpole development to provide a biological context where filopodia are important.

Which non-animal alternatives did you consider for use in this project?

Tissue culture cells and fruit flies.

Why were they not suitable?

Cells grown in dishes do not recapitulate the normal filopodia growth and movements, so do not directly replace the tadpole experiments, which themselves replace experiments with mammals. Our experiments with extracts cannot yet be done with tissue culture cells as the extracts that can be made are too dilute. Mammalian brain extracts have been used but these are not as effective as frog egg extracts. The microscopy of the filopodia in fruit flies is not as fast or clear to see as the frog nerve cell filopodia so we would be unable to answer the objectives for this particular project using flies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated based on our previous use and design of anticipated experiments over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Other licence holders have been able to superovulate frogs that lay good eggs 40 times, so we will do this to reduce the numbers of animals used. Bad layers are specifically identified by monitoring quality, rather than after an arbitrary number of superovulations. If bad laying frogs are identified we will kill them humanely.

We will keep frogs in salt solution for a day (if they suffer no visible harms from doing so) as they continue to lay and we can continue to use the eggs thus reducing numbers of animals used.

We use a low amount of extracts for our work by optimising dilution, using a small volume and carefully calculating the levels of reagents we need to use which reduces numbers of animals needed. We also perform pilot experiments to check we are using the correct conditions.

To obtain tadpoles, we fertilize eggs from 1 or 2 frogs at a time and use numbers of embryos that we are confident we will have time to process.

We use control experiments to ensure each experiment is meaningful, collect multiple datasets from each experiment and carefully perform steps to minimise variability.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We share the eggs and sometimes the fertilized eggs with other groups so less frogs are used. We track the quality of the eggs and kill frogs that do not lay good eggs rather than inject them again.

We will trial a double ovulation procedure, where each frog lays twice, which would reduce the number of animals by up to half if successful. We will only use this procedure if it does not increase the harms for each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The project uses Xenopus laevis, an amphibian model. We will induce the frogs to lay eggs and do this on repeated occasions as this is similar to what occurs in the wild.

The eggs will be used in two ways. One way is fertilizing them to make tadpoles that are used in immature larval forms before they are protected. The cells of the tadpole that will later become eyes are removed and cultured to obtain nerve cells. As a final approach we may image filopodia from cells directly within unconscious anaethetized larvae (before they are protected).

The second way is crushing the eggs in a test tube to make extracts.

Why can't you use animals that are less sentient?

The use of frog egg extracts is specialised and optimised for the field of study. Because frog eggs are so large they can be easily broken open without too much damage, leading to experiments that cannot be performed with other cell extracts. The filopodia produced by the larval nerve cells we work with have a known function in connecting the eye to the brain and so give us a meaningful setting for working on filopodia. The cells give very high quality microscopy and a biologically relevant setting which are not possible to achieve in any other system. The nerve cell experiments share important reagents with the experiments with extracts and the two sets of work are used in combination to gain the most information with least animal harm.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are transitioning to home-bred males and female animals to reduce the stress induced during traveling and adjusting to different environmental conditions. We keep handling to a minimum. All Xenopus are kept on dark backgrounds in environmentally enriched tanks. Using dark shelving and refuge tubes helps the animals to feel safe.

Frogs are monitored throughout the day while the technicians are in the room. If frogs appear stressed (excess skin shedding, overly jumpy, not eating) the NVS is engaged, and decisions are made as to whether or not it is best to humanely kill the frog. In the unlikely event a frog is in pain (a small number may develop a secondary bacterial infection colloquially called Red-Leg) the frog is closely monitored and if no improvement is seen within 48 hours the frog is humanely killed. Any frogs that have more that 20% body sores from diseases such as Red-Leg are euthanised immediately.

We work with a specialised Xenopus NACWO. All frogs are photographed for ID and health monitoring which is non- invasive and requires minimal handling, improving the clinical signs of the colony. We also monitor egg quality and keep the consistent good layers which reduces the overall number of animals held.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I am following PREPARE guidelines for planning experiments (https://norecopa.no/prepare) and ARRIVE guidelines to report my research (www.arriveguidelines.org). We will use published guidance on use of controls for using reagents that inhibit protein production (morpholino oligonucleotides) to ensure the best control experiments are used.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will discuss with my colleagues in the Xenopus field and consider practical guidance from the Named Veterinary Surgeon and Named Animal Care and Welfare Officer at my institution. I will refer to material from the NC3Rs website https://nc3rs.org.uk/resource-hubs, 3Rs tools in house and external resources such as Norecopa https://norecopa.no/databases-guidelines.

116. Standards in virology

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Vaccine, Influenza, Virus, Disease

Animal types	Life stages
Egg (fowl)	embryo, adult
Ferrets	Adult
Sheep	Adult
Rabbits	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

OBJECTIVES AND BENEFITS

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To generate biological materials that support global vaccine production and evaluation (quality control).

To provide biological reagents critical to (a) the World Health Organization (WHO)-led decision process for influenza vaccine composition; (b) global influenza vaccine production; (c) quality control testing of influenza vaccines for the global public

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Influenza vaccines are produced twice a year to meet the needs of the Northern and Southern Hemisphere flu seasons. To ensure the vaccines that are produced contain the types of vaccine virus most likely to give protection to vaccinated people, accurate characterisation of viruses is needed. This involves growing the viruses and making antibodies against them so that the vaccines can undergo quality control before being made available to the public.

Many vaccines need similar biological materials for an assessment of their biological quality - we will replenish expiring stocks with new for these vaccines and for new vaccines that become available.

This work is assuring the biological quality of human viral vaccines that are used globally.

What outputs do you think you will see at the end of this project?

The most immediate benefit is the continued availability of appropriately selected, manufactured and quality-controlled influenza vaccines for the world. Vaccines are produced twice a year to ensure availability for both the Northern and Southern Hemisphere influenza seasons.

The systems and assays used to control vaccines develop gradually over time but are well established and of demonstrated effectiveness. Currently reference materials and working reagents, which include candidate vaccine viruses and serum (containing antibodies against influenza virus proteins), are essential to the continued provision of potent vaccines worldwide including influenza vaccines. Such materials, which ensure the uniformity and quality of the vaccines concerned, must be available for both the Northern and Southern Hemisphere influenza seasons.

We expect to publish scientific papers in open access journals (i.e. available without the reader needing to pay) describing some of the studies that have been undertaken and the ensuing results including novel methodologies to best characterise the reagents, as well as any that in the future could reduce the reliance on the use of animals.

Who or what will benefit from these outputs, and how?

Ultimately, the benefits of the work in this licence is any individual in the world who receives an influenza vaccine or a vaccine for which the establishment provides reagents to measure its biological quality.

The provision of the reagents made under this licence contributes to the control of the potency and appropriateness of the vaccines produced by manufacturers and regulatory authorities and forms a central part of the process by which satisfactory and protective products are marketed globally. The consequences of using a vaccine of low potency or inappropriate strain(s) are that it will fail to protect the recipient.

The products that will be produced under this licence are a critical part of the standardisation and batch release of vaccines for a number of viruses.

For influenza virus, specifically, without the work covered in this licence, there would be a number of short term and immediate consequences:

- A lack of information to feed into the WHO-led influenza strain selection process, potentially leading to a poor vaccine match with currently circulating strains and therefore vaccine failure.

- A lack of new high growth reassortants could lead to a delay in the required number of doses being manufactured in the time available. These 'candidate vaccine viruses' may be used as the starting point for vaccine development.

- The licence supports work required for provision of the antibodies and antigen standards required to batch release (quality control) vaccines: without these reagents there would be an immediate effect on global vaccine supply. The antibodies are specific to different influenza types, or 'strains' and can therefore be used to confirm that the correct vaccine viruses are in the vaccine.

For other viruses there is a longer timeframe before replacement reagents are usually required, but a lack of reagents would also lead to potential vaccine shortage if batch release activities could not be undertaken due to these reagents not being available from the establishment.

How will you look to maximise the outputs of this work?

Staff contribute to WHO written technical monographs that support the process of influenza vaccine strain selection and vaccine quality control. Outcomes of the strain selection process are published on the

WHO website twice yearly. Reagents are made available to global stakeholders.

Whenever possible, scientists publish data in scientific journals - these are 'Open Access' so that anyone can access them free of charge online.

Species and numbers of animals expected to be used

- Sheep: 150
- Other birds: No answer provided
- Rabbits: 20

- Ferrets: 100
- : 2000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Embryonated eggs are the least sentient animal available. Most influenza viruses are grown in eggs - typically these will be less than 2/3 gestation and only on rare occasions would there be a need to grow a virus beyond this point when there are barriers to virus replication at the earlier stage of egg development.

Animals in which immune responses are required are selected as adults since very young animals may have an immature, and therefore incomplete immune response to the virus or other immunogen - this would possibly lead to a sub-optimal product.

Ferrets are among the few animals other than primates whose response to infection with influenza reflects that of humans; both the immune response and the clinical signs closely resemble that seen in humans.

Sheep are required to generate the large amount of serum required for a reagent for global use. Mice are too small and the response too variable to make them a practical alternative. Rabbits are used when only small-scale antibody production is required.

Fresh avian (here, turkey) blood has been identified as the most suitable for the haemagglutination (and inhibition) assays - to use alternatives would result in less reliable data and potentially greater volumes of blood from other species.

Typically, what will be done to an animal used in your project?

The studies require inoculations and boosters of antigenic material (including virus, virus components for example) with or without adjuvant into animals typically intramuscularly or subcutaneously, though

intranasal can be used for ferrets (may require up to 2 boosters for ferrets no less than weekly intervals; maximum of 6 boosters for rabbits typically weekly intervals; typically 3-6 boosters in sheep and on very rare occasions, up to 12 typically over the same number of weeks);

Subcutaneous implantation of a microchip into ferrets is optional to measure body temperature - it may be necessary to replace this once on the rare occasion that the chip is lost or fails.

Administration of antivirals orally to ferrets to ameliorate infection and symptoms due to infection by virulent influenza, and to reduce the likelihood of disease progression to the severity limit. Delivery will be twice daily for 7 days. The experiment will not typically exceed 3 weeks.

Blood collection from re-used animals. Blood collection from birds allows for re-use only after full recovery is demonstrated and with a minimum interval between collections: a maximum of 6 collections is permitted.

There is a possibility that viruses will need to be propagated in embryonated eggs at or beyond two- thirds gestation if the viruses cannot be grown to usable titres in eggs of less than this gestation time.

What are the expected impacts and/or adverse effects for the animals during your project?

General:

Typically the majority of animals used are not expected to experience significant ill-effects.

For any animal being immunised there may be some local irritation at the site of inoculations particularly where adjuvants are used (adjuvants are chemical compounds that increase the immune response to an inoculation). This will be monitored and advice from a Named Veterinary Surgeon will be sought if any adverse effect is seen. Exudative skin lesions at the site of inoculation are very rare, are expected to respond to local treatments and will not be allowed to persist for more than 24 hours.

Blood sampling procedures are expected to cause no more than minor transient discomfort. Very rarely haemorrhage, bruising, or haematomas may occur – the incidence of these will be minimised through good technique, use of needles of appropriate size and by applying continuous gentle pressure to the site after sampling. Single-use needles are employed. Maximum blood draw volumes will be adhered to and are based on Laboratory Animal Science Association (LASA) guidelines with expert input from NVS and Named Animal Care and Welfare Officers (NACWOs).

Any animals that are unable to readily feed or drink as a result of their condition will be humanely culled immediately. Any animal with deviation from normal health or behaviour which can't be alleviated with minor interventions such as those advised by the NACWO/ NVS within 24 hours will be humanely culled.

Eggs:

Where at, or greater-than-two-thirds gestation embryonated eggs are required, embryos that are adversely affected by the propagating virus may be terminated without delay. Embryo studies are completed prior to hatching thus adverse effects not be expected beyond 2-days.

Ferrets:

The adverse effects of influenza include rhinitis, fever and lethargy. Animals infected with virulent influenza viruses such as potential pandemic avian strains will usually develop fever, loss of appetite and reduced activity levels. Sneezing, nasal discharge, partially obstructed breathing due to mucous production and diarrhoea may also occur, as can be observed during human illness. Anti-viral drugs will be administered to ameliorate infection and symptoms. The virulence for ferrets of these viruses is variable and unpredictable, but if any symptomatic state persists for more than 24 hours, or an animal develops further signs of ill-health, action will be taken to minimise the adverse effects and suffering of the animal. There may be weight loss: a loss of >15% which does not appear to be resolving will result in the animal being terminated. Analgesic and anti-virals may be used but should it appear that humane end points are reached, the animal will be terminated to ensure no further suffering.

Turkeys:

The animals used in this protocol are not expected to experience significant ill-effects. Minor transient discomfort from the sampling may occur. Occasionally haematoma may occur at the sampling site but proficient technique and the application of local pressure after sampling will minimise the incidence. The wing veins of turkeys are very superficial and the blood vessel under the skin can be visualised quite clearly. Any haematoma or damage to the vessel is clearly visible. Past experience of blood sampling poultry has shown that any haematomas tends to resolve very quickly in a couple of days. An inspection of the vessels under the wing would clearly show any evidence of haematoma. In the unlikely event that a sampling site abnormality fails to recover; if it looked like there was no improvement between 48 to 72 hours; or the bird develops other signs of ill health the matter will be referred to the Named Veterinary Surgeon, whose clinical judgement and advice will be followed.

Sheep:

Irritation or muscular stiffness could occur at the inoculation site and this may result in lameness. If so this will be referred to the Named Veterinary Surgeon (NVS), whose clinical judgement and advice will be followed. In our substantial experience these occurrences are very unusual. Viral antigens administered by the intramuscular route are not expected to induce adverse systemic effects and in our experience these have never been observed or reported.

Rabbits:

The use of a specific adjuvant in rabbits has been reported to be associated with the risk of developing granulomas approximately 1 week post inoculation. To minimise this risk, the inoculum will be spread over 4 inoculation sites to to minimise the inoculum volume at any one site. The areas of inoculation are depilated by clipping to improve accuracy of

inoculation. Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The overall severity under this licence ranges from Mild to Moderate depending on the Protocol.

Ferrets approx. less than 50% of ferrets will experience moderate severity levels; typically no more than 30 animals over the lifetime of the PPL.

For the ferrets that will be infected with virulent influenza, the severity may be moderate. Since the outcome of these infections can be unpredictable the animals will be monitored very closely for signs of, reduced food intake, reduced water intake, lethargy, and will be treated with antiviral medication to limit the symptoms they experience due to the infection. Any ferret displaying neurological clinical signs, which may include symptoms such as ataxia or seizures will be culled immediately

Avian eggs - all Mild;
Turkeys - all Mild;
Rabbits - all Mild;
Sheep - all Mild

What will happen to animals at the end of this project?

Killed
Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Specific antibodies are required for use in tests to characterise viruses and evaluate viral vaccines. These are complex and need to have a broad activity against the viruses and vaccines candidates that can mimic what might be observed in higher vertebrates - humans. Because of the complexity of the mammalian immune system, these can only be generated in protected animals. The same complexity and quality cannot be achieved in a non-animal system currently.

Research into technologies such as phage display may ultimately be able to replace the use of animals. For the studies outlined in this licence, however, the application of alternative methodologies is still in development. The validation of a method is complex since it needs to involve generation of antibodies that can effectively mimic the polyclonal ("broad") response that is generated by animals to various influenza proteins and candidate vaccine viruses.

These then need to be in volumes sufficiently large that global laboratories and vaccine developers can use them to the volumes required in their processes. The approach would then need to be validated in other laboratories to ensure a harmonised approach globally. Thus the projects are still very much in a research phase and the use of protected animals to provide the required antibodies will need to be continued for the foreseeable future. If a new technology looks to become a viable alternative, the use of this will need to be tested alongside the current methods to prove its validity before the use of animals can be replaced.

The use of antibodies produced by in vitro methods is under investigation for use in influenza vaccine potency assays. If validated this would remove the need to produce antibodies in sheep for new influenza vaccine strains.

Which non-animal alternatives did you consider for use in this project?

The use of reverse genetics (RG) to prepare reassortant viruses allows for precise engineering of desired reassortants. This is an alternative to classical reassortants produced in eggs. Reassortants prepared by RG do not require rabbit antisera to eliminate undesirable reassortants, although the use of ferret sera for antigenic characterisation of the resulting viruses is still a requirement, and the RG viruses are still grown in embryonated eggs. However, use of these viruses for vaccine manufacture is hampered by restrictions associated with intellectual property rights (IP) unrelated to the Establishment

– it is unable to force manufacturers to use viruses generated by reverse genetics as this would be a conflict of interest, due to promoting the use of a patented technology which is owned by one particular company.

Materials prepared for vaccine manufacture and standardisation of vaccines must be prepared on the same substrate as vaccines. Propagation of virus in embryonated eggs is currently the most common way by which vaccines against influenza are manufactured. While the use of cell lines is increasing, there is still limited use for generation of standard reagents. However, with an increase in the number of cell lines approved for isolating viruses, vaccine development and production there exists the possibility of removing the need to use embryonated eggs in the future.

Turkey red blood cells are critical components of haemagglutination (HA) and haemagglutination inhibition (HI) assays used to assess viruses and viral vaccines and the immune responses they elicit. There is no reliable commercial supply of turkey blood of

suitable quality. In order to be able to isolate red blood cells of appropriate quality it is essential that blood is drawn from live turkeys, blood obtained post-mortem is not suitable. To kill a turkey each time red blood cells are required would be an extreme measure. Birds are monitored for adverse events as a result of blood sampling (such as haematoma formation) and only bled again under re-use authority if they are fit and healthy with no sign of adverse reaction to previous blood sampling.

As above, cell lines might be used in the future, depending on the wider vaccine production field.

Why were they not suitable?

Tissue culture is not a commonly used substrate for influenza vaccine production, limiting its use at the establishment for generation of standard reagents. However, more recently cell lines have been approved for isolating viruses, vaccine development and production and this affords the possibility of removing the need to use embryonated eggs in the future. These have limited utility currently until the wider vaccine field moves away from egg-based vaccines completely.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers reflect previous years' use and allows for flexibility should a number of influenza strains need to be changed in any one season - typically this would be 1, perhaps 2, but there may be instances where additional changes are made or replacement reagents (particularly serological) are essential to ensure vaccine supply.

The number of eggs required is predictable and is regarded as the minimum to assess the material needed to test vaccines. The volumes of blood from turkeys are those required based on years of experience of undertaking the specific laboratory tests.

Few animals are required to generate the sera that are needed, but experience has shown over the years that several animals must be immunised or infected to be sure that there are sufficient suitable sera. Typically, up to six ferrets will be used in infection experiments and the same number of sheep for immunisation with non-replicating antigens. The numbers of animals proposed is regarded as the minimum necessary to produce the desired result, based on the experience of many years.

Influenza viruses are sufficiently different that it is not easy to predict how good an immune response an individual animal will make - we pool sera collected from multiple animals to ensure supply and homogeneity of the samples.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The numbers are based on previous experience. The experiments do not change substantially between iterations of this project licence since they are linked into the global approach to influenza vaccine production.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The numbers of animals used under the Protocols will be the minimum required to obtain sufficient biological materials – viruses, antiserum – as based on historical scientific data and experience with the species.

The individual project plans will include a pre-study meeting to identify the number of animals required per group, in cases where the experiment has not been previously undertaken.

We maintain dialogue with vaccine manufacturers to determine if (and when) they can use less volume of sera to test their products, and if so we will be able to reduce the amount we provide and thus reduce the number of sheep used to make sera.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Embryonated eggs - this is the least sentient species; monitoring of eggs in which virus is propagating in the allantoic fluid will minimise suffering of embryos which can be terminated early should there be signs of infection of the embryo itself. The use of eggs at or past the two-thirds gestation will only be necessary should the virus under investigation be empirically shown not grow to the titre in eggs of less than two-thirds gestation needed for onward use. If eggs at or beyond two-thirds gestation are required, the eggs will be inoculated after setting, and allantoic fluid will be harvested by or at Day 17. The use of

eggs past the two-thirds gestation period is a rare requirement and was not needed in the previous PPL.

Ferrets - infection with influenza viruses and generation of small volumes of antiserum. In order for the ferrets to seroconvert they must be actually infected with the influenza virus, which can lead to clinical signs, and be kept alive long enough for antibodies to be produced by B cells. For animals infected with virulent influenza we will use antivirals to reduce the clinical effects of infection: animals will be given antivirals by the oral route twice daily for 7 days beginning 2 days following an initial intranasal administration of antigenic material. It is recognised that group housing is preferable for optimum well- being of ferrets and wherever possible they will be group housed. The use of remote measurement of body temperature will minimise the amount of animal handling required and thus keep stress levels to a minimum.

Rabbits - generation of antiserum; we have refined the method of delivery of adjuvanted antigen during the course of a parallel and current PPL to minimise the risk of granuloma. The use of a specific adjuvant in rabbits has been reported to be associated with the risk of developing granulomas approximately 1 week post inoculation. To minimise this risk, the inoculum will be spread over 4 inoculation sites to to minimise the inoculum volume at any one site. The areas of inoculation are depilated by shaving to improve accuracy of inoculation.

Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin. By adapting the inoculation process the likelihood of granuloma is reduced and thus the potential for lasting harm is likewise minimised. Should granuloma form, it is possible to pause the protocol until the animal recovers sufficiently, and then select an alternate site for inoculation if needed.

Rabbits will be housed in socially compatible groups whenever possible and always in floor pens.

Sheep - required to generate the large volume of serum required for a reference antiserum reagent for global use. The effectiveness of alternative adjuvants has been and will continue to be explored with the aim of minimising or removing the need to use Freund's adjuvant while achieving the required quality of the antiserum in vaccine manufacture. Currently, Freund's complete adjuvant is required for the initial (prime) inoculation only, whereas Freund's Incomplete adjuvant is used for the subsequent (boost) inoculations. The inoculations in sheep are managed by expert staff to minimise distress of the animal through the inoculation and blood collection procedures; there is availability of food and/or grazing after the procedure for the animals. The sheep are habituated and maintained together as a group. The procedures are not anticipated to result in lasting harm over the duration of the study.

Mice are too small and the response too variable to make them a practical alternative to the sheep.

Turkeys - fresh red blood cell supply is critical for accurate and reproducible in vitro tests. The availability of the blood from the second establishment close to the primary establishment allows for such supply. As blood ages post collection, critical red blood cells can lyse resulting in more variable scientific data which may require additional samples to be used. Here, blood is collected from a bird from a flock which is habituated and kept together over the period of many months. To minimise the impact of blood withdrawal on any one bird, the birds are selected on a cycle system to allow sufficient periods of rest in between blood collections and minimise lasting harm. No single bird is used more than once every 21 days and typically it is at an interval of 42 days.

For rabbits, ferrets and sheep: analgesics will be administered where needed and where their effect will not interfere with the scientific outcome of the study.

All animals will be killed humanely at the end of the experimental protocol / study by the method in the relevant Protocol.

Why can’t you use animals that are less sentient?

Embryonated eggs are the least sentient animal available: the use of eggs at or greater than two-thirds gestation is for those exceptional, though not readily predictable, influenza viruses that do not propagate to usable titres in eggs of less than two-thirds gestation.

Ferrets are among the few animals other than primates whose response to infection with influenza reflects that of humans: both the immune response and the clinical signs closely resemble that seen in humans. Methods for more frequent observation of clinical signs have been developed for earlier recognition of onset of disease allowing earlier intervention with the use of medication to relieve symptoms or termination as appropriate. Data accumulated over the years indicate that the specific immune response is highly predictive for when a strain is sufficiently different from those that have gone before to warrant a strain change in the vaccine. The relationship is far less clear-cut for other species such as mice where infection is usually more restricted.

Rabbits are suitable for small volumes of polyclonal antisera. To generate the same amount of serum in rodents would require substantially more animals.

Sheep are required to generate the large amount of serum required for a reagent for global use. Mice are too small and the response too variable to make them a practical alternative.

Most studies last a number of weeks therefore terminally anaesthetised animals are not a practicable option.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All animals are habituated prior to commencement of study.

The use of remote telemetry (microchip) of ferrets is based on previous experience to minimise impact of regular temperature monitoring.

Inoculation volumes are based on those recommended by LASA and others; they are the minimum required at any administration site. These have been shown to be appropriate over the previous licence.

We have made changes to practices under the previous PPL such as refinement of inoculation of rabbits to minimise the risk of granuloma formation. To minimise this risk, the inoculum will be spread over 4 inoculation sites to to minimise the inoculum volume at any one site. The areas of inoculation are depilated by shaving to improve accuracy of inoculation. Attention is paid to delivering inoculum with no seepage from the needle prior to inserting needle and on removal, thereby minimising irritation to the skin. Reinoculation (boost) of rabbits that do develop granuloma can be paused until the animal is considered recovered.

Freund's complete adjuvant is used for the initial inoculation of a sheep only. This is currently the 'gold standard' for generating anti-influenza hyperimmune serum in sheep. In line with published scientific literature we employ a schedule in which FCA is used for the first inoculation, but not subsequently, since it has been reported that no increase in immune response was seen with continued use of FCA.

Neither Freund's Complete nor Incomplete adjuvant is used in other species in this project. We will continue to review the scientific literature around adjuvants used, and communications with scientific and veterinary groups to determine whether there could be suitable alternatives to FCA (and others) that can be immunogenic but reduce the reactogenicity in animals. Should credible candidates be identified, a comparative study of these against existing adjuvants (particularly FCA in sheep) can be considered with suitable amendment to the Licence to ensure good experimental design and statistical power.

We have a programme of continual improvement to animal housing, husbandry and handling aimed at minimising stress in animals and encouraging natural behaviours.

Animals undergo thorough pre- and during-study checks. Analgesics will be administered where needed and where their effect will not interfere with the scientific outcome of the study. Clinical signs can be ameliorated by the use of antivirals (ferrets) or analgesia (ferrets, rabbits, sheep), for example.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance is available through Home Office including newsletters via the ASRU.

LASA and FELASA guidelines will be applied to guide dose volume/route per laboratory animal subject and surgical procedures.

NC3Rs documents on husbandry, housing and procedural work will be met or exceeded.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Establishment's AWERB circulates up to date information on the 3Rs through the Named Information Officer (NIO). Similarly, the licence applicant is on the email circulation list for the NC3Rs bulletins.

The PPL holder will work closely with the NACWOs and NVS to ensure that changes to the animal studies are improved wherever possible. This may be reflected in changes to husbandry and animal handling practices, refined procedural activities, and advances reported in the scientific literature or at scientific conferences/meetings that could result in use of a less sentient species, fewer animals in experimental groups, or non-animal alternatives.

Information exchange will be used to refine the work programmes as necessary.

117. Ecology and epidemiology of infectious disease in wildlife

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - (Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Wildlife, Epidemiology, Demography, Management, Disease

Animal types	Life stages
European Badgers	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The over-arching aims of the study for the next five years are to collect demographic, behavioural and epidemiological data from wildlife populations, in order to construct mathematical models and conduct statistical analyses to identify trends in the dynamics of infectious diseases and responses to management interventions. The involvement of wildlife in the persistence of infectious disease in livestock presents a serious challenge to its control. The findings of this study will be used to inform the evidence base available to develop options for the sustainable management of the risks of onward transmission of infectious disease from wildlife to livestock.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many pathogens of wildlife can cause serious disease in humans and domestic animals. Effective management of transmission risks and control of disease outbreaks requires an understanding of the ecology of the wildlife host and the epidemiology of infection in host populations. This study will focus on infectious disease of UK livestock and a major animal welfare issue which imposes significant costs on the taxpayer. Data from this project will inform the development and implementation of sustainable approaches to manage risks of transmission between wildlife and livestock. The study will also provide opportunities to investigate other infectious diseases that may circulate in wildlife and/or transmit to humans and domestic animals (e.g. coronaviruses, tick-borne diseases and salmonella).

What outputs do you think you will see at the end of this project?

Pathogens of wildlife can infect humans, domestic and companion animals, imposing a significant financial and social burden on UK society. In the past, data from our studies has provided important insights into the dynamics of infectious disease in wildlife and livestock and has been instrumental in influencing policies for the management of infectious disease. Data from our studies will continue to be used to further develop mathematical models used to assess different control strategies and will continue to be used to inform disease management approaches. Specific outputs expected during the lifetime of this project will include evaluation of methods of marking vaccinated wild animals and taking blood samples for trap-side diagnosis, and detailed observations on the effects of culling on badger behaviour and disease transmission. This information will be particularly valuable when embarking on a policy of vaccinating animal populations that have previously been subject to culling.

Who or what will benefit from these outputs, and how?

Over the course of the next 5 years this project will provide information of particular relevance in the control of infectious disease risks from wildlife. In addition to furthering our broad understanding of the drivers of the spread and persistence of this disease, we also anticipate specific outputs that will inform plans to roll out wildlife vaccination in areas previously subjected to culling. Our studies will therefore contribute to the ongoing control of a disease of livestock which is a substantial burden on farmers, the UK taxpayer and a welfare issue for livestock and wildlife. Being a zoonotic infection and is therefore of concern for public health.

In the short-term the outputs of this study will inform development of approaches to implement vaccinating wildlife in post cull areas, and in the longer-term will contribute to the wider goal of disease eradication in UK livestock.

How will you look to maximise the outputs of this work?

Outputs from the project will be maximised through communication with policy-makers (so they may inform policy development), collaboration with academia, joint publication in scientific journals, presentations at national and international meetings, and dissemination of findings to key stakeholders including the farming industry and their representatives, and conservation groups.

Species and numbers of animals expected to be used

- : 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Wild animals of all age groups can be infected with disease and can be involved in the persistence of infection in livestock. During our field studies we will collect fine scale information on the dynamics of this disease, the demographics of wild populations, and their social behaviour (contact patterns in particular) and how these relate to disease transmission and attempts to manage disease spread.

Typically, what will be done to an animal used in your project?

Under this licence an animal will typically experience being captured in a trap and transported to a sampling facility where it will be anaesthetised for examination and sampling. On first capture each animal is given a number and letter combination tattoo on the belly for future identification and a hair sample is taken to provide a unique genetic identifier. At each capture animals will be visually examined to assess their age, sex, condition and reproductive status, they will be weighed and their body length measured. A series of samples will be taken to establish whether they are infected with disease, including blood, sputum, urine, faeces and swabs of any wounds or abscesses. Some captured animals may have skin scrapings, additional nasal, oropharyngeal or anal swabs taken or ticks removed, in order to investigate other parasites and diseases. A small number of animals will have tissue biopsies taken from the ear to investigate ageing and whiskers may be collected from animals to investigate foraging behaviour. Collars carrying tracking devices will be fitted to some animals to allow their movements and contact behaviour to be tracked. After sampling all animals will be allowed to recover fully from anaesthesia. Animals will typically be held for up to 24 hours (up to a maximum of 36 hours if required) prior to release back into the wild. Once deemed fit for release each animal will be transported back to where it was caught and set free. Animals may experience this process several times in their life.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected level of severity of procedures is mild. No lasting adverse effects are expected as a result of capture, examination or monitoring of wild animals. Nevertheless, we have detailed procedures in place to address any welfare issues arising from unexpected outcomes. At the end of the study animals will be set free into the wild. We observe a closed season when animal trapping is suspended (Feb – Apr) to avoid catching lactating females and dependent young. As all captured animals are only away from their social group for a short period of time we do not anticipate any significant impacts on their social position. The observed social stability of the population we have been studying since the 1970s indicates that our activities have not caused any noticeable social disruption.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild for all procedures.

What will happen to animals at the end of this project?

Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Complete replacement of the use of live animals is impossible as the purpose of the research described here is to investigate their role in the persistence of infectious disease and options for managing disease risks to livestock. However, the data available from our studies can be used in simulation models to investigate the likely effects of different management scenarios and the dynamics of disease under other conditions, which may reduce or obviate the need for the use of live animals in the future.

Which non-animal alternatives did you consider for use in this project?

Disease modelling was considered.

Why were they not suitable?

This is a study of disease in wildlife which provides basic biological data that can be used to build models that simulate disease dynamics and responses to management interventions. However, these models can only provide limited information on the relative outcomes of different management approaches and cannot answer fundamental questions about the disease in wildlife and their behavioural responses to infection and management interventions. It is also necessary to use live animals in order to field test new management techniques (e.g. trap-side blood sampling) and diagnostics. Hence, at present no non-animal alternatives exist for collecting the data required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This is a study of wild animals and so it is not possible accurately forecast numbers that may be captured as this will vary between populations and according to weather and local circumstances. In addition, as this study adapts to the requirements for evidence to inform disease control policies, it is not clear in advance how many animals might need to be captured to answer specific questions. However, the figure of 750 animals is based on numbers that have been captured in recent years working under the previous project licence, as this provides the best estimate of numbers likely to be required over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

These studies are largely observational, involving long-term monitoring and sampling of wildlife populations, and so there is no formal experimental design as such. However, where sub samples of animals are required to test new methods or answer specific research questions then we will take statistical advice on experimental design and use power analyses to determine how many animals to recruit into these studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The numbers of animals in this work will be optimised through the use of highly skilled field staff and very effective means of capturing wild animals, which ensures that our field operations are efficient (under a range of environmental conditions) and that we do not lose any captured animals. Also, the skilled personnel who carry out anaesthesia and all procedures ensure that we obtain a full set of samples from virtually every animal. The use of highly experienced staff and standard operating procedures results in very few adverse

effects being observed and allows us to obtain high quality samples. Our in-house expertise in mammal ecology and infectious diseases, and collaborations with specialists from academia maximise the value of the data obtained from the captured animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The study requires capture, examination, sampling and monitoring of wild animals in which infectious pathogens circulate. The research team have considerable experience in the trapping and handling of wildlife, they have continued to develop and improve field and sampling methodology in the interests of minimising animal suffering. In the light of experience, trap design has been refined to reduce the likelihood of injuries. The team includes a veterinarian and full time NACWO. All methods are described in operating procedures and all staff undertake extensive training, including annual refreshers on techniques and direct supervision. Trapping is carried out under licence and each study within this wider programme of work is subject to scrutiny by the AWERB Panel.

Why can’t you use animals that are less sentient?

Replacement of animals with a less sentient species is not an option as the purpose of the research described here is to investigate natural ecological and epidemiological processes in wildlife populations.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our working practices have been refined over many years but are the subject of continual improvement. Procedures have been refined on the basis of emerging evidence and at our annual refresher session we run through existing procedures with all staff involved and provide an opportunity for all to raise any issues/suggestions for improvement. We encourage a culture of shared responsibility for animal welfare in the team and an openness to discuss any concerns or ideas for improvement.

Some recent refinements are,

1. The gradual replacement of traps with a mesh size that is associated with a lower likelihood of injuries during trapping. This was achieved by systematically monitoring injuries (most of which are very minor, e.g. skin abrasions) associated with different types of trap over time.

2. The introduction of additional measures to monitor body temperature of animals under anaesthesia so we can pick up departures from normality and take appropriate action when required.

3. Completely emptying drinkers immediately prior to the administration of anaesthesia of animals in holding cages, so as to avoid any animal becoming unconscious with its snout in the water.

4. As part of work involving deploying radio/GPS/proximity logger collars on animals, we implemented a procedure of getting each newly fitted collar double checked by another experienced person.

5. The use of camera traps to locate collared animals in order to target trapping effort and so maximize chances of recapturing them and removing the collar.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In my role as PPL holder I stay up to date on innovations in wildlife research to identify opportunities to improve our working practices. This involves staying informed about the latest literature and in particular any best practice guidance. Our AWERB Panel also pass on any published best practice guidance that may be relevant.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I undertake ongoing CPD (often identified by our local AWERB panel) that is relevant to the 3Rs and my role as a PPL holder. In addition, close liaison with the NACWO, NVS and NIO provide further opportunities. I also personally stay informed about advances in the 3Rs in wildlife research through my regular access to the scientific literature. I am able to consider the implementation of any advances through discussion with the team involved in the study, the NACWO, NVS and NIO. Our annual refresher session provides a useful forum for this and allows all staff working on the project to raise any issues. I am able to authorise changes to the working practices on the project as I am the lead scientist for this area of work in addition to being the PPL holder.

118. Mechanisms of metabolic regulation in health and disease

Project duration

5 years 0 months

Project purpose

- Basic research

Key words
cancer, metabolism, obesity, diet, imaging

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The primary goals of this project are to study how tumours change the metabolism of the host organism, and how, in turn, metabolic changes in both tumour and the host support tumour development.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite significant progress in understanding the molecular basis of how tumours emerge and develop, cancer remains a significant cause of death worldwide. In particular, liver cancer, a major focus of this project, is the fifth most deadly tumour type, with no substantial improvement in patient mortality rates over the past three decades, in contrast

to most other tumour types. The rise in liver cancer cases is, partly, due to an alarming increase in obesity-induced liver disease, which is typically associated with accumulation of fat and subsequent injury in the liver. Chronic liver injury, in turn, promotes cancer - almost all liver cancer patients have had some form of liver disease earlier in life and about 20% of patients with cirrhosis (an advanced form of liver disease, also associated with excessive alcohol consumption) will develop liver cancer. However, there are no clear indicators that predict which patients with liver disease will go on to develop liver cancer. Furthermore, there are limited therapeutic options and, even then, it is unclear which patients will respond to these therapies. Therefore, clinicians need to observe patients with liver disease over long periods of time in order to capture those that do develop cancer early, when surgical resection still offers hope for survival. Beyond the cost in human lives, such long-term patient monitoring adds a significant cost to health systems.

A common feature of cancer cells is that they change their metabolism, compared to normal cells, in order to survive and proliferate. Targeting cancer metabolism offers significant therapeutic potential as evidenced by the fact that the first chemotherapy, methotrexate, targets a metabolic pathway and is still successfully used today in the clinic. However, there is a high failure rate in producing effective drugs that target metabolism, in part because there are many gaps in our knowledge about basic metabolic processes. Specifically, it is unclear how changes in metabolism of the host organism, which are often found in patients with various types of cancer, help tumour initiation, maintenance and dissemination (metastasis). Alterations in the metabolism of the host, such as loss of fatty (adipose) tissue and muscle mass, also have profound effects on the quality-of-life of cancer patients and, in part, contribute to mortality.

The proposed work brings these two challenges under one umbrella by aiming to provide mechanistic insights into how tumours change host metabolism and how host metabolism, in turn, supports cancer formation, in order to identify new therapeutic strategies; and apply these insights into testing new therapeutic approaches for liver cancer. Some of the metabolic pathways we study play important roles in normal physiology and our studies will also provide insights into these normal functions. Finally, by using models other than liver cancer, such as mammary gland cancer, we also aim to explore whether our findings have broader relevance for oncology.

What outputs do you think you will see at the end of this project?

Broadly, we expect to produce new knowledge about the role of specific metabolic processes in normal body physiology and tumorigenesis, and to generate new genetic, chemical and computational tools that will accelerate mechanistic understanding of the functions of metabolism in health and disease. More specifically:

- We will elucidate the mechanisms that tumours employ to alter the metabolism of the host organism and will assess whether and how these pathways can be targeted for cancer therapy.

- We will generate new genetically altered mouse lines that harbour knockouts of metabolic enzymes to assess the role of these enzymes in normal physiology, in metabolic disease and tumorigenesis.
- We will develop new small animal imaging methods to measure dynamically metabolic pathway activities in living animals and validate their usefulness as a tool to monitor disease progression and response to therapy.
- We will validate small molecules that alter the function of metabolic enzymes to assess their value as potential therapeutics against cancer.

- We will use our data from animal experiments to generate and train new computational models of animal physiology and metabolism that have improved predictive capacity.

The new knowledge acquired from our work will be distributed through a wide range of channels including presentations and posters at relevant conferences, and publications in relevant journals. In addition, we will also announce breakthroughs and updates through social media channels such as Twitter and the institutional website.

Who or what will benefit from these outputs, and how?

- Basic science and the scientific community: Metabolism underlies all aspects of life and is linked to the biggest killers of human society, incl. diabetes, infectious diseases and cancer. Research into the metabolic basis of disease is rife and our work will provide important insights into the fundamental mechanisms mammals use to maintain a healthy metabolism, how these mechanisms are perturbed in disease and how we can intervene to restore a healthy outcome. Our work will also generate new genetic, chemical and computational tools that can be used to interfere with metabolic processes. In the short term, both the knowledge and tools generated from our work will be available to other researchers to be used and inform their work.

- 3Rs: In the longer term, our work holds great promise for helping to reduce the number of animals required for future metabolic research. Our proposed work will help us train computational models of mouse metabolism to improve their ability to predict which metabolic processes to target in order to stop tumour growth. In turn, better computational models will help us refine our hypotheses and design more targeted experiments that will enable us to achieve our scientific objectives using a smaller number of animals. Similarly, once validated, our imaging methodologies have the potential to either decrease the numbers of animals needed to obtain similar insights, or obtain more data-rich results from each animal without repeated sampling.

- Clinical translation/patients: Ultimately, we envision that our work will benefit human patients by improving our understanding of cancer’s impact on whole body metabolism. Liver cancer patient mortality has not improved for decades, in contrast to other tumour types. Patients with cancer of diverse primary origin suffer both due to perturbation in the affected tissue’s function but also due to disturbance of systemic metabolism, which leads to frailty and poor quality-of-life. Our work on the metabolic consequences of

tumorigenesis, will illuminate new strategies towards ameliorating whole- body physiology and to halt tumour growth. Beyond our focus on liver cancer, our work will also test whether whole-body metabolic changes are also relevant in other cancers, such as breast cancer, and in metabolic disease.

How will you look to maximise the outputs of this work?

A major goal of this project is to understand how tumour-induced changes in host metabolism occur and their role in tumorigenesis. Host metabolism alterations have been reported in patients with tumours of various tissue origins. So, although most of the planned work focuses on liver cancer, we are also proposing targeted experiments in other mouse cancer models to assess how our findings apply to other cancers, thereby expanding the scope of our work's translational applications.

Dissemination of newly acquired knowledge and experimental tools will also help maximise our work's outputs. We will report our findings to the broader scientific and clinical community through publications in scientific journals and presentations in national and international conferences. Newly generated tools, such as imaging protocols, genetic knockouts and computational models of mouse physiology will also be made available through appropriate channels, such as mouse and software depositories.

These tools will catalyse further research by other researchers world-wide.

The proposed work will also facilitate existing, or instil new collaborations both with academic and industrial partners. For example, our mouse metabolism data will help to improve computational models by mathematical biologists that would, otherwise, not be able to readily test their models' capabilities. In turn, computational models will help us process large amounts of data and generate new, testable hypotheses. Furthermore, our newly discovered metabolite analogues with improved pharmacokinetics may be of interest to other academic or industrial partners who seek to use in vivo validated chemical tools to study the consequences of perturbing metabolic pathways in mice.

Species and numbers of animals expected to be used

- Mice: 48450

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Most human metabolic pathways and the processes that regulate them are highly conserved in rodents making the mouse the lowest animal in the evolutionary tree that is appropriate as a model species to study metabolic disease and cancer, the main focus of our work. In practice, mice have proven invaluable in elucidating mechanisms that

maintain an organism in a healthy state. Consequently, mice have also been a cornerstone of preclinical drug evaluation for most successful therapies. Furthermore, there are numerous available genetically modified mouse lines and refined procedures that can significantly accelerate testing new hypotheses into molecular mechanisms and alleviate the need to re-develop experimental approaches that would necessitate additional animals.

Given that cancer significantly affects ageing populations, most experiments will be performed in adult mice (typically up to 40 weeks of age). Metabolic deterioration during ageing (e.g. age-induced inflammation and diabetes) may contribute to higher tumour incidence in older individuals. Therefore, to assess whether metabolic pathways in the host that support tumorigenesis change as mice age, we will also perform metabolic analyses in older adults (typically approx. 1-1.5 years old). Conversely, some of the metabolic enzymes we study have been implicated in inborn errors of metabolism affecting neonates and children. Therefore, mice harbouring genetic deletion of these genes will be valuable models of these metabolic diseases. In such cases, in a small number or animals, some metabolic measurements will be performed in a small number of neonate or juvenile animals.

Typically, what will be done to an animal used in your project?

1) Generation of mouse models of cancer and metabolic disease.

In general, we will generate mice that develop tumours by various genetic or chemical methods. In most cases, mice will also be fed diets high in sugar and fat, which are known to cause obesity and are linked to increased human cancer incidence. In some cases, mice will undergo surgical procedures,

e.g. to remove an endocrine organ that is implicated in producing tumour-promoting signals. All surgical procedures are relatively short (up to 30 minutes).

2) Monitoring metabolic body parameters and tumour growth.

Most animals will be scanned by non-invasive imaging methods (primarily MRI) to monitor tumour development, metabolic parameters or response to therapy. Imaging will be done at regular intervals throughout tumour development, and up to the time that tumours approach the humane end point. In cases where response to therapy is assessed, tumour monitoring may be extended for up to a time required to confirm no re-emergence of therapy-resistant tumours.

3) Measurement of metabolic activities in vivo.

To measure metabolic activities in tissues of living mice, most experimental animals will be fasted between 6-18 h and then administered with labelled metabolites. The metabolic state of some experimental animals will be assessed by administering a single dose

(bolus) of a metabolite (e.g. glucose) or hormones (e.g. insulin) followed by sampling of a small volume of blood.

4) Testing effects of pharmacological or genetic modulation of target pathway upon tumour development.

In some cases, to test whether a metabolic process is needed for tumour emergence or development, an agent that alters the activity of such a process will be administered over a period of time that is long enough to observe an effect upon tumour growth. Suitable compound doses, administration routes and frequencies will first be determined in pilot experiments. Substance administration will not exceed predetermined volume and frequency limits that are approved in this licence.

What are the expected impacts and/or adverse effects for the animals during your project?

Experimental procedures proposed for this project have either been established or will be refined to minimise the possibility of adverse effects. Animals that develop tumours may experience weight loss, appetite loss, hunching, or temporary shivering. Animals will be monitored daily and weighted weekly, and any animals experiencing more than 15% weight loss between these measurements will then be weighed daily and killed if approaching the humane endpoint. Notably, in some cases weight loss during therapeutic treatment regimens may be associated with improving, rather than deteriorating, overall health, e.g. when animals are switched from an obesity-promoting to a normal chow diet.

Objective assessment of whether weight loss is a cause of concern will be aided by assessing the overall body condition and behaviour of animals (e.g. for signs of distress or piloerection).

Surgical or chemical damage to the liver is reversible and full liver mass and function are expected to recover within 6-7 days after treatment. Surgical procedures and cell injections can cause internal bleeding. Animals are closely monitored during procedures and directly after recovery and any animal showing evidence of bleeding will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Moderate (60-70%)
- Mild (30-40%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To identify mechanisms of growth and metabolism that are relevant in the context of the whole intact animal and are therefore meaningful for human clinical studies, it is necessary to utilise animal experiments. This is because, organ- and cancer-specific metabolic pathways are highly influenced by the complex physiological processes inside the body, the local tissue microenvironment, and metabolic crosstalk between different organs and tumours; none of these features are yet possible to faithfully recapitulate in vitro.

Which non-animal alternatives did you consider for use in this project?

Our current and future research makes extensive use of alternatives to animals. In vitro cultures of mammalian cells/tissues, under conditions made more physiologically relevant as informed by the experiments described in this project, can be used for some of our studies that focus on cell- autonomous mechanisms and are undoubtedly an important source of replacement.

We are also making extensive use of computational models of mouse metabolism, whereby, metabolic processes that are predicted to be important for cellular functions related to cancer (such as biomass production to support proliferation). To provide meaningful predictions, these models require input from prior experimental measurements but can be used to test or refine hypotheses before experimental validation in animals.

Why were they not suitable?

The main aim of this project is to understand how organs interact to maintain whole-body metabolic homeostasis, the perturbation of which is a hallmark of many severe diseases such as cancer, infectious disease and metabolic syndrome. It is, therefore, fundamentally impossible to study this problem in any other model than the intact animal. In addition, the effects of manipulations of the diet can only be meaningfully studied in vivo.

Furthermore, cultured cell systems alone are inadequate to address most of this project's aims because they cannot recapitulate the metabolic input from the gut and tissue microbiomes; they cannot mimic circadian effects; they lack the multitude of cell types found within different tissues and overall tissue architecture (known as "zonation" in the liver). All these parameters are known to have profound influence on cell metabolism.

Finally, although our improved computational models are increasingly making better predictions that help refine experiments and reduce animal numbers, they need to be validated experimentally before making any meaningful decisions about the translational potential of our discoveries.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Most experimental models proposed in this project have already been established in the lab. We therefore have a clear picture of assay limitations in the context of the expected variability from animal physiology and we can empirically estimate how many animals each experimental group requires for our studies. For new methods, we have consulted the literature and colleagues with relevant experience.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our animal studies design is aided by the NC3Rs Experimental Design Assistant and uses the PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines. We consult with a statistician with regards to the experimental design to minimise the number of animals used whilst ensuring meaningful data can be collected.

We always strive to invent, improve or implement novel technologies to obtain more information from each animal. Then we incorporate those techniques that are suitable to obtain the desired output while minimising animal numbers at strategic points in our experimental plan. For example, when we need to study a specific pathway (rather than the entire metabolic network, which requires specialised measurements in tissues from terminal experiments) we use magnetic resonance spectroscopy (MRS) to obtain dynamic metabolic activity measurements in a tissue-specific manner. This approach provides, from a single mouse, data that would otherwise require dozens of animals. We use MRS to measure lipid content in a spatially resolved manner without killing the animal, which can then be used for other measurements. For therapeutic treatment studies, longitudinal MR imaging (MRI) ensures cohorts have the desired tumour burden, negating the need for increased animal numbers that would otherwise be used to offset therapeutic response variability due to diverse starting tumour size, or to avoid losing animals that unexpectedly reach the humane end point before the completion of the study.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Before embarking on any animal experiments, we will collect as much evidence as possible to determine whether a candidate genetic or environmental manipulation has a reasonable chance of regulating mammalian growth and metabolism in vivo. Evidence will

be collected from our own initial studies using cancer cells cultured under physiologically relevant conditions, as well as by surveying the relevant literature. In addition, we will use non-regulated procedures to collect expression data from fixed non-GM mammalian tissues and functional/expression data from genetically and/or environmentally manipulated in vitro mammalian cell lines.

Where possible, mouse lines will be maintained in a homozygous state, thereby obviating the generation of a large excess offspring with inappropriate genotypes. In other cases, homozygotes will be generated from heterozygote inter-crosses, with littermates genotyped as heterozygous or wild type used as age and gender matched controls. In the case of our liver studies, when scientifically justified, we will make use of viral vectors with tropism to the liver (e.g. adeno-associated virus serotype 8 – AAV8), to decrease the need for breeding multiple alleles to obtain tissue-specific genotypes.

For most of the quantitative experiments, design will be based on PREPARE guidelines. Otherwise, we will use the minimum number of animals to provide an adequate description of the projected outcome, determined on the basis of previous experience (our own, or from the literature).

This programme of work will make optimal use of several tissues, fluids and cell types per individual mouse. We will aim to collect organ samples from multiple body sites and to provide other affected tissues to appropriate scientists, so that they do not have to breed mice specifically for their experiments. This highly integrative approach will maximise the information obtained from the minimum resources. Cryopreservation of gametes, embryos, tissues and cells is routine at our establishment and will ensure that the minimum number of mice is bred.

We also expect that, as we gain better understanding of the metabolic processes involved in biological systems under study, we will be able to define more tailored methods to investigate metabolism while maximizing the amount of information derived from each animal, e.g. by defining new biomarkers or in vivo imaging probes that would allow longitudinal observations of metabolic functions and thereby reduce animal numbers. This is likely to have application not only for this project but also for the broader research community.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use mice to study inter-organ communication in health and disease because, although relatively low in the evolutionary tree, their overall anatomical distribution of metabolic functions and metabolic network organisation are highly similar to those in humans. Furthermore, there are many genetic tools and highly refined techniques available for mice, which aid scientific discovery.

The genetic and chemical carcinogenesis models of liver and mammary gland cancer are well established, are known to have relatively minimal adverse effects and have been extensively characterised in many labs around the world. Furthermore, the selected liver cancer and tissue damage models, alongside diet manipulation, recapitulate specific aspects of human disease, that, collectively, contribute to morbidity.

Why can't you use animals that are less sentient?

One of our primary scientific goals is to understand how organs communicate to achieve whole-body homeostasis, and less sentient species, such as the fly, do not recapitulate the overall anatomical and functional distribution of metabolic activities across organs (e.g. the fly tissue that is thought to be the liver equivalent only performs some of the mammalian liver functions, in addition to other functions that are performed by the mammalian adipose tissue). Most of our measurements with metabolic tracers are done under terminal anaesthesia. However, for studies on cancer growth and metabolic disease development, both of which are intimately linked to ageing, and develop over several months, we need to use adult mice as this is the most physiologically relevant life stage.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Wherever appropriate, we will minimise the adverse effects associated with genetic alterations by using inducible or conditional alleles to delete gene activity from specific tissues rather than from the entire mouse. To minimise stress during breeding and maintenance, we will follow best practice guidelines and follow local refinements of husbandry such as cage enrichment and sufficient amounts of nesting material. On receipt or generation of a new line, we will minimize suffering by ensuring increased observation and monitoring until a detailed phenotypic analysis for each line is accomplished. If any welfare implications are identified, they will be acted upon and refinements considered in consultation with the NVS and NACWO.

For all manipulations we will adhere to local or national guidelines that aim to minimize suffering. Many of the genetic, dietary, temperature and oxygen manipulations as well as the administrations of gene inducers/repressors or other agents are standard and previous refinements from the literature will be used. If, however, there is insufficient information available, new manipulations will be pre-screened in small-scale pilot studies to obtain

indications of the minimum dose and exposure time that is likely to be effective, thereby minimising any potential suffering.

In all surgery, analgesia will be provided according to contemporary best practice and advice from the NVS/NACWO. In work done under a previous licence, we have optimised surgical techniques, such as partial hepatectomy, to accelerate the procedure, minimise incision size and reduce the probability of collateral tissue damage. Good aseptic surgical techniques, heat & fluid therapy will be provided. In the case of cancer models for example, we will follow the guidelines in Workman et al, British Journal of Cancer (2010) 102, 1555 – 1577. For each protocol where there are a number of optional steps, the maximum number of steps is clearly defined within the adverse effects section.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidance given in the NC3Rs 'Resource Hub' (https://nc3rs.org.uk/resource-hubs) for example on blood sampling (https://www.nc3rs.org.uk/blood-sampling-mouse) and effective use of genetically altered mice (https://www.nc3rs.org.uk/GAmice). We will also refer to the National Cancer Research Institute guidelines on using animals in cancer research published by Workman et al. 2010 (British Journal of Cancer 102, 1555 – 1577).

For surgery, we will follow the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/wp-content/uploads/2018/05/Aseptic-Surgery.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will keep up to date with the latest developments on refining animal research methods via the NC3Rs website (https://www.nc3rs.org.uk), also complemented by information we obtain from regular newsletters prepared by our animal facility. Animal house staff will ensure that any advances are fully implemented throughout the facility.

119. Neural circuits in health and disease

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Behaviour, Depression, Neural circuits, Neuronal cell types, Opto- and chemogenetics

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to study connections between nerve cells (neurons) using mice under normal healthy conditions and in an animal model of depression. Neurons make connections with each other and our aim is to access these connections between nerve cells in various brain regions that are shown to be altered in depression. These neuronal connections will subsequently be manipulated using state- of-the-art chemo- and optogenetic methods that are modern neuroscience techniques in which genes for chemical- and light-sensitive proteins are introduced into specific types of brain cells in order to monitor and control their activity.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The brain is the most complex and least understood organ in the brain. Brain diseases include a wide spectrum of disorders including depression. More than 20 million people in Europe suffer from depressive disorders and overall costs that comprise of both direct costs (including medication and hospitalisation) and indirect costs (that are (i) morbidity costs that include wages lost by people who are suffering from depression and are thus unable to work and (ii) mortality costs that incorporates costs due to lost life-years after premature death) were calculated to nearly 120 billion Euros in 2004 (Sobocki et al., 2006).

Scandalously, over 30 percent of patients are considered to be treatment-resistant (Otte et al., 2016) underpinning the urgent need to develop novel treatment options. Of note, the female:male ratio of patients suffering from major depressive disorder is approximately 2:1 (Albert, 2015). The latter stresses the need to perform basic and translational research taking the gender into account (Schiebinger, 2014).

In order to develop novel treatments, it is further important to better understand the role of various peculiar subtypes of neurons including excitatory pyramidal neurons and inhibitory GABAergic interneurons in both health and disease.

With this work we will gain (1) insight into the neuronal connectivity between different brain regions known to be involved in depression, and (2) how these connections are altered during depression in a gender-specific fashion.

This will not only lead to additional information on how neurons communicate with each other, but also on the mechanisms of depression that will be useful for other researchers working on depression. Our findings might provide an avenue of developing new targets for treating this devastating disease.

What outputs do you think you will see at the end of this project?

The major output of this project will be new information on the mechanism of how depressive-like behaviour emerges and a better understanding of the brain areas and neuronal cell types being involved.

Findings will be published in academic journals and presented on conferences to disseminate our research results. The data will be made available in public databases to increase accessibility and reproducibility.

Who or what will benefit from these outputs, and how?

This work will contribute to generating novel knowledge on the structure and function of the healthy brain and how neuronal networks are changed in models of depression.

Although a new treatment might not be the direct result of this study we will provide valuable information that can be used to guide the development of new treatments long-term and help patients suffering from depression in the future.

How will you look to maximise the outputs of this work?

We will disseminate our findings by presenting them at national and international conferences and by publishing scientific research articles. We aim to publish the data in open-access journals, which has been shown to widen the audience.

We will also report negative results and try to explain why these experiments may have failed as it is generally agreed on that other scientists can still learn from negative results studies.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using mouse models. The primary reason for using mice is the ease of genetic manipulation of this species and the ability to obtain relevant mouse models from appropriate repositories. In addition, there is an extensive literature using this species to investigate underlying causes of neurological and psychiatric disorders. The use of genetically altered animals (GAAs) allows us to study cell-type diversity and the role of specific cell types in neural circuits in health and disease. There are currently no feasible alternatives that would replace GAAs in order to perform these investigations.

Typically, what will be done to an animal used in your project?

Some mice will be exposed to a depression-inducing protocol known as maternal separation with early weaning. Mice of this group, and also of an age-matched control group, will undergo surgery to inject viruses into specific areas of the brain. Injected mice recover well from the surgery. Subsequently, mice be tested for depressive-like and reward-seeking behaviour in behavioural tests such as the sucrose preference test, the splash test or the open field test.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice might experience pain after surgery. They will be closely monitored. To minimize adverse effects local analgesics will be given perioperatively.

The depression protocol might have adverse effects, both during maternal separation, and following weaning. Possible adverse effects that may be encountered during maternal separation include hypothermia, and rejection by the mother upon return to the home cage. To avoid hypothermia, mice are isolated in a cage on top of a heat mat and provided with plenty of bedding. To avoid rejection by the mother, mice are handled with gloves to mask experimenter odour and rubbed with bedding from the home cage to re-familiarise them to the mother. The main adverse effect that may be encountered in mice exposed to maternal separation is that they might not gain weight as they would otherwise. To ensure that pups are strong enough to undergo the separation, litters of no more than 8 pups will be used. Litters will be provided with baby food from the last two days of maternal separation till a week after weaning. Littermates will be kept together for first week of weaning to allow them to share body heat and keep each other warm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Stereotactic surgery: moderate

Maternal separation: moderate

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Brain connectivity and the effect of stress on brain development needs to be studied in a living animal. There are currently no in-vitro alternatives to study these objectives.

Which non-animal alternatives did you consider for use in this project?

We are constantly reading the relevant scientific literature to look for relevant alternatives and also check relevant databases. At the current state of knowledge, the most promising technology is in vitro brain organoids. These are groups of cultured neurons that can be encouraged to develop into the rough organisational formation of a brain in a Petri dish. However, this technology is currently very primitive, and while we hope to utilise this in the future, this currently does not recreate the level of organisational complexity required to reproduce and study psychiatric symptoms such as depression.

Why were they not suitable?

The human brain contains nearly 100 billion nerve cells and even in small animals the central nervous system is comprised of millions of neurons that are precisely wired together. Proper neuronal wiring is achieved during development under optimal conditions that involves coordinated steps from neuronal growth to accurate matching of neural connections. To date, mimicking these conditions in a dish has not been accomplished making it difficult to study neuronal circuits both under normal conditions, but also in disease.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated, with the help of the institutional biostatistician, based on statistics from preliminary experiments, statistics from our own published and similar works, and experience from the previous PPL.

A maximum 500 animals will be used over the two-year application. They will be bred under Protocol 1 and then either be kept for replacement breeders or move to other protocols within the licence for use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have performed power calculations based on data previously generated in our lab, which gives an indication of the potential effect size between control and test groups. The numbers included in this application are the minimum numbers required to reach statistical significance for each experimental group, and as such the minimum numbers required to detect an effect.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Taking our experience from the previous project into account we consider efficient and well-planned breeding as a crucial measure to optimize and reduce the number of animals in this project.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will be using the maternal separation model of depression. Separating mice from their mothers from day 6 until day 16 for 6 hours a day into individually isolated compartments with early weaning at day 17 cause stress and has been shown to result in a depressive phenotype. After weaning mice will be group housed where possible to reduce further harm and they will only briefly be separated for tests that need the mouse to be single housed such as sucrose preference testing. During this time mice will have nesting material and a little house in their cage at all times to minimize distress to the animals.

Why can't you use animals that are less sentient?

It is not possible to study higher integrated processes such neuronal information processing using cell- based assays, or simple model organisms (e.g. yeast) that lack the required neuronal pathways to allow a detailed understanding that can be translated to human physiology.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mice exposed to maternal separation are at risk of hypothermia during maternal separation, and being rejected by the mother upon return to the home cage. Additionally, they might not gain enough weight to thrive after early weaning. To avoid these possible adverse effects during maternal separation, pups are kept in a cage with a heat mat and lots of bedding, and are handled with gloves and rubbed with home cage bedding upon returning to the home cage. To ensure the pups thrive following weaning, litters of no more than 8 pups will be used. Litters will be provided with baby food from the last two days of maternal separation till a week after weaning. Littermates will be kept together for first week of weaning to allow them to share body heat and keep each other warm.

All animals in recovery experiments may experience some post-operative pain or discomfort because of surgery. Animals will be monitored on showing signs of adverse effects, like their coat state and how they are behaving. We will use post-operative analgesia to reduce surgical post-operative discomfort.

Health condition including body weight, spontaneous movement, head/face swelling, and appearance will be monitored and scored in the first several days after surgery, and

treatment interventions (such as analgesia or food/heat support) and/or humane endpoints will be applied as per conditions in the licence.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the appropriate websites such as NC3Rs refinements, NORINA, relevant literature and breeding information.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will receive email newsletters from the 3Rs websites and we will discuss these issues with colleagues regularly to stay informed. We will also receive relevant information from our NIO and regularly liaise with NACWO and NVS.

120. Tau pathology in alzheimer's disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Tau tangles, Selective cellular vulnerability, Genetic modifiers and risk factors, Clearance pathways, Alzheimers disease

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged
Rats	embryo, adult, pregnant, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify why tangles form and dementia occurs, and how to prevent or treat it.

Dementia caused by clumps of the protein tau is seen in several dementias including Alzheimer's disease (AD) and FrontoTemporal Lobe Dementia (FTD-tau). The tau clumps have been termed Neurofibrillary Tangles ('tangles') by pathologists. Tangles are therefore a significant contributor to neurodegenerative disease and dementia. The overarching aim of my research programme is to understand the mechanisms that cause tangles to form, especially in AD and FTD-tau, focusing on mechanisms involved in what goes wrong in the brain, why there are so many different diseases that have tangles as the root cause, and the impact of tangles on brain function, including the type of memory and learning

problems ('cognitive impairment') that we associate with dementia. All aspects of the work are attuned to the potential for therapeutic targeting and intervention. Our work aims to identify how the genetic make-up of the mouse (in parallel with studies on humans) influences whether you will get tangles and dementia, and how severely; mechanisms driving the formation of tangles and dementia and potential therapeutic interventions; why certain brain cells are more vulnerable than others to the harmful effects of tangles. It is hoped that deeper understanding in these research areas will drive the development of therapeutics for people living with dementia.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tangles form in the vast majority of people who have dementia as they are a major factor causing both Alzheimer's disease (AD) and frontotemporal lobe dementia (FTD-tau), and more than 20 other diseases or syndromes that present with dementia. AD accounts for 60-80% of all dementias and FTD- tau accounts for approximately 50% of all FTD cases which in turn accounts for 10-20% of all dementias. Tangles are also seen in patients with traumatic brain injury and following certain microbial infections. Alzheimer’s disease is characterised by the accumulation of two types of proteins - tangles made of tau and also plaques made of a protein called amyloid. Although amyloid seems to occur first and drive tangle formation, loss of neurons and memory decline is linked to tangles forming. Over the past decade, it has emerged that after tangles first form, the tau protein that causes them spreads across the brain from neuron to neuron, causing worsening of the dementia that accompanies it. As part of my new research programme and using a series of sophisticated methods, I hope to find ways in which to stop tau accumulating in tangles and spreading across the brain and thereby prevent or slow disease development in people.

What outputs do you think you will see at the end of this project?

This project will result in new data for the science community to use, publications, presentations and possibly new drug therapies to combat dementia.

Who or what will benefit from these outputs, and how?

People with dementia, especially those caused by Alzheimer's disease or Frontotemporal dementia (FTD-tau) will benefit from these outputs. The research proposed aims to identify what causes these diseases by focusing on one of the causative proteins, tau, which can accumulate in brain cells and kill them. Our research aims to identify why the toxic protein tau accumulates in the brain, how it injures and kills brain cells and how this leads to the memory loss and ultimate death of people with this diseases. We also aim to test whether we can treat the disease in mice that have engineered to develop the diseases. Outputs

such as presented or published data will be beneficial to the scientific community in the short term; therapies or new targets are not expected to be fully realised until completion of the project.

How will you look to maximise the outputs of this work?

My lab has a long history of collaboration to accelerate and streamline research and through rapid dissemination of new knowledge through presentation and publication and through the provision of resources (eg. intellectual knowledge, lab resources) to others. This includes the deposition of new mouse models to repositories such as Jackson Labs.

Species and numbers of animals expected to be used

- Mice: 30,000
- Rats: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use rodents for the research, especially genetically altered rodents that mimic Alzheimer's disease and FTD-tau. New developments in the field have generated good models of dementia that can be used to test hypotheses as to why people get dementia, and ultimately to test therapies. There are no suitable alternative animal models for the disease that reproduce dementia. We will examine rodents at young and old life stages to ensure we reproduce the life stages relevant to dementia.

Typically, what will be done to an animal used in your project?

Many of the mice on this licence will be used for breeding. This will allow us to achieve aim to understand how particular genes cause neurodegenerative diseases. Several steps of breeding are required to get to a cohort with the necessary combination of genes that can be studied.

In order to understand specific pathways implicated in tau pathology and the aetiology of Alzheimer's Disease, some animals will undergo short (5 min-maximum 1 h) procedures where either expression of specific genes and/or proteins will be altered or certain substances that will modulate synaptic and/or cellular function will be applied at appropriate life stages of the animal. These procedures may be minimally invasive (eg. dosing of drugs via injection or oral gavage to test how interventions achieve our aims to identify disease causing mechanisms, or possible treatment) or more invasive (eg. intracranial injection to directly measure brain functionality). Tissues may then be harvested from the animal at a designated point of pathology development.

Animals may additionally or alternatively undergo surgeries to implant cranial apparatus permitting in- vivo brain imaging, or direct stimulation of specific brain regions. This will allow us to measure brain function and test whether normal brain function can be rescued. Anaesthesia and analgesics will be given as appropriate to the procedure, and careful monitoring and humane endpoints will be applied to all animals. The maximum number of cranial surgeries experienced by an animal within its lifetime is limited to 3 procedures.

A subset of mice will undergo a combination of phenotyping and/or behavioural tests to characterise preclinical models and for hypothesis testing. This is important as the diseases we are studying cause dementia and severe behavioural problems therefore we need to be able monitor cognitive performance in the mice.

At the end of an experiment, typically animals are deeply anaesthetised and a terminal bleed or perfusion carried out.

What are the expected impacts and/or adverse effects for the animals during your project?

Genetic alterations in the mice used in this project may lead to the development of dementia-relevant phenotypes. In mice, these changes may lead to effects such as: abnormal behaviour including increased aggression/fighting activity, mild motor impairment, seizure-like activity, or decreased food intake leading to weight loss or altered response to some procedures.

For most animals, the duration of these effects will be limited. Animals displaying moderate severity of their phenotype that is detrimental to their welfare such as seizure behaviours, weight loss, or significantly abnormal behaviour, will typically be used for an experiment within 1-2 weeks of onset of these moderate effects, or sooner if so advised in consultation with the NACWO and NVS. Where animals show a milder phenotype that causes little impairment to their well-being, the duration of these mild effects may be prolonged (eg. 6 months - 1 year). Care will be taken to use animals at the soonest timepoint where the desired phenotype is expressed, to limit the duration of any suffering.

For some tests, mice will need to undergo anaesthesia. All surgery and anaesthetic use in mice carry a risk of mortality and a risk of pain (short and long term), which may differ between genetically altered lines. Appropriate analgesia will be applied to all animals undergoing surgical procedures (or other non-surgical, painful procedures) to minimise any acute suffering.

Mice undergoing cognitive testing using a food reward for motivation will have reduced access to food to reduce their body weight by ~10% from their free feeding weight. Studies have shown that in some mouse models that are genetically modified to have dementia-causing disease, food restriction increases survival, consistent with data for C57BL/6 mice in general. Thus, we do not anticipate adverse welfare outcomes associated with food restriction. To improve the validity of our results we will monitor long-term effects of food

restriction by assessment of blood glucose before and after food restriction and body mass composition.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 5% of animals may experience 'non-recovery' severity, as they will only be subject to procedures under terminal anaesthesia. This is limited to wild-type animals undergoing terminal cardiac perfusion. They will therefore be returned under a 'non-recovery' category.

A further 20-30% of animals will experience only sub-threshold severity, as their genetic alterations will not cause any adverse phenotype.

Approximately 25% of animals may experience mild severity, either through the severity of the phenotype caused by their genetic alteration, or due to the nature of the procedures applied to them (such as non-invasive injections of non-harmful substances).

The remaining 40% of animals may experience moderate severity. These animals will typically be those entered onto protocols involving a surgical procedure, or may be animals that have been bred to exhibit a moderate phenotype and display adverse effects such as seizure behaviours, abnormal behaviour, or decreased body weight.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Alzheimer's disease and FTD-tau afflict a very large number of people, especially the elderly, and they manifest as dementia. While some research in this field can, and will be done on cell models, ultimately the only way to test whether we have really understood what causes dementia, and how we should treat it will need experiments to be performed in animals as they have a complex brain that can replicate the memory aspects of humans. For example, people in the earliest stages of Alzheimer's disease get lost easily, even in familiar environments. They have problems with spatial navigation.

Using mice that have been genetically altered to develop Alzheimer's disease, and sophisticated within-brain recording and behaviour testing techniques, we are working on identifying particular cell populations in the brain that are responsible for the problems in spatial navigation. We are also working on rescuing the deficits in brain circuitry that are linked to these problems in a way that could be impactful for AD patients. These experiments would not be possible in humans, or animals with less complex brains.

The primary objectives of this project are to:

- Link molecular and cellular changes of dementia-causing diseases to dementia-relevant phenotypes (including changes to cognition, behaviour, metabolism, sensory systems, body fluids, tissues and whole-body physiology).

- Understand how environmental changes and pharmacological interventions can modulate dementia- relevant phenotypes. It would be unethical (and not possible) to test humans with dementia with interventions that have not been approved by regulatory agencies.

It is not possible to manipulate the molecular and cellular pathways in people who have dementia in order to determine how these processes impact cognition, behaviour, metabolism, sensory systems, body fluids, tissues and whole-body physiology. Thus, an animal model must be used. We need to use an animal model to manipulate pathways and we cannot observe humans directly as we need to begin before obvious symptoms manifest, and throughout the course of the disease, taking many different measurements (some invasive) to see how they relate to each other. The wider research community is undertaking significant work analysing clinical and human post-mortem datasets from people who had dementia-causing diseases, but these only give a 'snap-shot' of what is happening in the brain, and it is usually only performed post-mortem, reflecting the very end stage of disease. We also perform preclinical work in cell, organoid and invertebrate model systems of dementia-causing diseases.

However, to fully understand all the factors causing dementia we need to use well defined and reproducible animal models, and manipulate specific factors associated with the disease to tease apart how they interact with the complex environment of the brain.

This project will link with and draw on these experiments to minimise animal use and maximise translational value of the preclinical mouse work in this project. The work in this project cannot be addressed elsewhere using alternative approaches.

Which non-animal alternatives did you consider for use in this project?

Clinical longitudinal studies of people who have dementia (biomarkers including blood, CSF and neuroimaging), genetic and cognitive/behavioural/clinical scoring.

Human post-mortem and brain biopsy studies.

Induced pluripotent stem cells (iPSCs) and direct conversion induced neurons from human patient fibroblasts, brain organoids, transfected HEK cells

Computational models

Why were they not suitable?

Clinical longitudinal studies of people who have dementia, provide correlative data between an individual's genetics, environment, biomarker (blood or cerebrospinal fluid biochemistry, or brain imaging data) and cognitive and behavioural outcomes. These datasets can be used to generate hypotheses of the molecular and cellular causes of dementia-associated biology but cannot be used to test how specific factors influence dementia outcome. For example, we cannot look at people pre- clinically (before symptoms of disease) and monitor the disease as it starts and progresses. We cannot genetically manipulate people and see how different genes impact disease. We cannot administer different pharmacological or neuromodulation interventions that are not approved for human use, and test which work, and how. We cannot record how well individual cells and brain circuits are functioning while the person is alive.

Human post-mortem and brain biopsy studies comparing people who had dementia (early and late in disease course) with people who did not have dementia, can be used to determine which cellular and molecular changes occurred in the brain. In some cases (especially AD which is more prevalent, but not FTD which is a relatively rare disease), clinical and biomarker data from life will also be available for these individuals and this observational data can be used to hypothesise which cellular and molecular changes resulted in clinical disease. However, these samples cannot be used to directly test hypotheses, and work out which hypothesis is correct without testing in animal models. For example we cannot tell whether neurones or immune cells are responsible for dementia, or which cellular pathway causes degeneration, and whether that is responsible for dementia.

Similarly, iPSCs and brain organoids cannot currently be used to study cognition, behaviour, metabolism, sensory systems, body fluids, tissues and whole body physiology.

In addition to non-animal models, we also considered using invertebrate models of disease. Invertebrate (fly) models can be used to study the response of neurons to some cellular pathways, for example, the proteins that misfold and aggregate in dementia-causing diseases. However, flies do not have complex brains capable of sophisticated behavioural and cognitive abilities, thus it is not possible to study how the cellular and molecular processes that result from an event such as protein misfolding impact the brain cell populations and circuitry leading to the executive function or subtypes of memory that go awry in dementia. For example, it is not possible to quantify working memory, motivation or apathy in a fly. Flies also lack key brain-resident immune cells (microglia) and a closed circulatory system (veins and arteries), which play key roles in the development of neurodegenerative disease.

These aspects, and many others involved in the biology of dementia cannot be modelled in flies.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The programme leader has more than 25 years of experience with AD or FTD-relevant mouse models and has published more than 70 papers describing experiments in mice similar to those proposed here.

Current estimates are based on our prior knowledge of the fecundity and attrition, timing and robustness of disease characteristics and variability seen in the type of mouse models proposed. Factors driving the speed at which pathology develops include transgene expression levels. Variability is impacted by genetic background. Based on pilot and

published date, we estimate that we will need a sample size of 10 per sex and genotype for each phenotyping experiment.

In view of the total number of animals used under in previous animal projects, the new plan of work and expected size of my team (10-15 researchers), we estimate that we will require approximately 10,000 mice for transgenic breeding, maintenance of colonies, and animals subject to no procedural interventions where post-mortem tissue is used in experiments. It is estimated that multiple rounds of breeding may be required to generate animals carrying multiple genetic alterations for experiments, and we anticipate providing other researchers with cohorts of mice matched to those studied for collaborative phenotyping projects

We estimate that approximately 1000 rats are to be used as pups or embryos for phenotypic analysis at specific stages of developments.

We anticipate that a further 20,000 mice may be entered on to the subsequent phenotyping protocols: current estimates are that we will need a sample size of 10 per sex and genotype for each experiment.

No reuse of animals is planned.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Power equation calculator will be used for group size calculations for all experiments plus attrition rate calculation will also be used to ensure sufficient power is maintained until the end of longitudinal studies.

We have additionally used the NC3Rs Experimental Design Assistant within the planning process of our upcoming experiments, to inform our expected cohort numbers and ensure that sufficient power is maintained.

The combination of tests in each experiment will be designed to gather the most meaningful data. Tests which can inform each other will be carried out on the same mouse to remove inter-animal variability and increase the power, thereby decreasing the overall sample size and the scientific utility of generated data. This approach will also provide novel scientific insight into the relationship between dementia-relevant phenotypes and help validate new testing approaches such as home cage assessment.

SOPs have been written and used routinely for previous projects. This standardises the way the data is collected and reduces the variability and therefore the sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have selected to use state-of-the-art animal models that best replicate human dementia so that our data will be valid, relevant and reproducible in the future.

Where possible we have selected models that require the least breeding (genetically homozygous mouse models) to generate sufficient animals for studies, and ones that reproduce the disease in a shortened timespan. Lines will be bred efficiently and holding lines as frozen-down embryos and sperm will be used to minimise the number of mice being produced for these studies.

Before undertaking experiments in animals, we will have performed as much of the work in cell and computational models as possible.

We have coordinated with other researchers to share tissue resources and results so that we will limit the number of animals used as much as possible. Any excess stock will be offered to other researchers to minimise wastage.

Genetically modified lines will be sourced from repositories to avoid remaking of lines whenever possible.

Pilot studies will be undertaken to generate means and standard deviations for work using background strains for which data is not available. Tissues sampled from the animals used in this project will be shared with other researchers and the data produced linked to that generated by the project, to maximise long term utility.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will largely use transgenic or otherwise genetically altered (eg. knock-in, knock-out) models of Alzheimer's disease or fronto-temporal dementia. Additional reporter lines (eg. Cre-ERT2 models, fluorescent protein models) that do not display any adverse phenotype will be interbred with AD mice.

We have selected to use state-of-the-art animal models that best replicate human dementia so that our data will be valid, relevant and reproducible in the future.

All animals models used are ones that reproduce the disease phenotype in as short a timespan as possible to limit the suffering of the animal. Wherever possible, we propose to principally use preclinical models that have a reduced welfare burden and provide a more refined scientific tool.

Why can't you use animals that are less sentient?

Experiments have been designed to use animals that are less sentient as much as possible. For example, we will be able to perform some of the research in brains from mouse embryos.

Where possible, terminal procedures will be performed preferentially to recovery procedures. However, some animals will be required to undergo chronic procedures (eg. repeated procedures of in-vivo brain imaging), or procedures wherein time is then required for pathology to develop or be characterised.

Moreover, we will assess how neuroimmune pathways lead to dementia-relevant diseases (including cognitive and behavioural changes); this will focus on the molecular and cellular biology that underlies these changes to cognition, behaviour, metabolism, sensory systems, body fluids, tissues and whole body physiology.

Invertebrate (fly models) can be used to study the response of neurons to the proteins that misfold and aggregate in dementia causing diseases and to screen for genetic modifiers of these processes.

However, they do not have complex behavioural and memory biology or complex neuroimmune system and thus it is not possible to study the cellular and molecular processes that result in altered executive function or subtypes of memory in dementia, as in this project. For example, it is not possible to quantify working memory, motivation or apathy in a fly. The fly cardiovascular system significantly differs from that in mammals (being open without veins or arteries) thus the effect alterations to cardiovascular biology on dementia cannot be readily modelled in a fly. This work needs to be undertaken in an adult model organism with an intact nervous system, blood-brain-barrier and robust immune response without anaesthesia to assess disease-causing pathology in their brains.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Experiments will be designed to balance the overall experience of the mouse and the number or type of tests undergone by any one animal against the value of a full understanding of the biology of individual animals and why phenotypes are altered in order to maximise utility of the research data obtained.

Mice undergoing phenotyping tests have increased monitoring (defined per protocol) and are removed from tests if they appear to be suffering from an adverse stress reaction, or other unexpected adverse effects of the phenotyping tests.

When general anaesthetics are necessary, the combinations with least potential for adverse effects will be used. Mice which have undergone anaesthesia have extra monitoring until fully recovered and extra checks when back in the holding rooms. Peri-operative analgesia will always be provided, and animals will be checked post-operatively by the responsible PIL and the animal facility technicians (see protocols for defined schedules of checking per procedure step). In case of any post-surgical complications, or any unexpected adverse effects, NVS advice will be sought.

For any experiments that may induce stress in the animals, care will be taken to reduce stress through gentle handling, habituation to equipment or behavioural arena, or training according to best-practise guidelines.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Routes and volumes for administration of substances are taken from Laboratory Animal Science Association good practice administration of substances guidelines 1998 (http://www.procedureswithcare.org.uk/lasa_administration.pdf).

Standard operation procedures for most tests have been generated using data and expertise from multiple animal houses and can be found at https://www.mousephenotype.org/impress.

ARRIVE and PREPARE guidelines will be followed at all times.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We (lab and PI) will attend the NC3R annual conference; we will also attend international meetings focusing on animal models of dementia causing disease and webinars run by the MODEL-AD consortium. In addition we will attend local 3Rs seminars and events, as the NC3Rs liaises closely with the Institute and University.

121. Supply of materials to develop and maintain tests for transmissible spongiform encephalopathies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Testing, Diagnosis, TSEs, Material, Collection

Animals Types	Life Stages
Cattle	juvenile, adult, aged
Sheep	juvenile, adult, aged
Goats	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To support diagnosis and eradication of naturally occurring transmissible spongiform encephalopathies (TSEs) in ruminants by ensuring that material is available for test development and validation.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

TSEs are notifiable diseases in the UK, which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE, "mad cow disease") in cattle and chronic wasting disease in deer. The BSE agent is the cause of variant Creutzfeldt-Jakob Disease in humans, which has so far affected more than 170 people in the UK. Eradication of TSEs in farmed animals is a global objective as it impacts on human and animal health and affects trade. There is a legal requirement to monitor susceptible animals for TSEs. Detection of disease relies on reporting of suspect cases or active monitoring of a subset of slaughtered or found dead animals, which then need to be diagnosed and confirmed by postmortem tests. These tests need to be validated, and testing laboratories need to be regularly quality assessed to ensure adequate diagnosis, which requires appropriate testing material. In addition, material may also be required to develop further tests or inform on the level of infectious agent contained within the tissue, which may be important for food safety if the tissue is edible. Furthermore, there is the legal requirement to maintain familiarisation with the clinical presentation to aid in the education of livestock owners. This work aims to fulfil these requirements by either acquiring animals suspected of having naturally occurring TSEs or exposed to naturally occurring TSEs or by experimentally infecting animals if naturally occurring case numbers are too low or not suitable to acquire the necessary material.

What outputs do you think you will see at the end of this project?

The output will be the generation of material needed for TSE test development and validation, for distribution to other diagnostic laboratories for external quality assessment (EQA) so that they can continue providing a diagnostic service, and to address research questions requiring study of th disease in the natural host. Depending on the research questions, this may result in publications or data input into risk assessments. In addition, it may generate training material about the clinical presentation to aid in the reporting of clinical suspects in the field.

Who or what will benefit from these outputs, and how?

It is a legal requirement to carry out TSE surveillance, and all processes in relation to the diagnosis need to adhere to quality standards. TSE tests require validation, and laboratories require regular validation and quality control to be able to carry out surveillance. Training material generated through observation and examination of animals will support passive surveillance. Without surveillance, the United Kingdom will be unable to trade in ruminant livestock and their products with other countries.

TSE studies in the natural host using material from these animals will aid in addressing important policy questions, e.g. assessing distribution of the infectious agent may inform on safety of edible tissues for human consumption and potential spread of the infectious

agent in the environment. This is beneficial to consumers and policy makers to protect human and animal health.

How will you look to maximise the outputs of this work?

Although material generated through this licence is to be used for domestic purposes, various tissues will be archived for future use or research requests from other countries. In the past, tissues have been distributed to various research institutes, which often require positive control tissues.

The output of any research addressing policy question will be beneficial both nationally and internationally, e.g. EU-wide if included in opinions of the European Food Safety Authority working groups.

Species and numbers of animals expected to be used

- Cattle: 22
- Sheep: 250
- Goats: 250

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All animals used will be the natural host for these diseases since tests have to be carried out and validated in tissues from the relevant species. Due to the long incubation period, the majority of animals will be adults or reaching adulthood when developing clinical disease if experimentally inoculated or naturally exposed.

Typically, what will be done to an animal used in your project?

Blood will be collected from all animals prior to euthanasia and tissues collected post mortem.

Naturally infected animals will be acquired and transported to the research establishment for clinical examination, which may include additional clinical tests to support the clinical diagnosis or rule out alternative diseases. Due to statutory requirements and restrictions to the source farm it is not expected that clinical suspects will be kept longer than a week.

Animals naturally exposed through contact with infected animals but without clinical disease and confirmation of disease status will be kept for longer to study disease development, which may include examination of a rectal biopsy taken under local anaesthesia to inform on disease status.

Experimentally infected animals will usually be inoculated with TSE brain homogenate under general anaesthesia and analgesia and monitored for disease development, which could take months or years.

What are the expected impacts and/or adverse effects for the animals during your project?

Study end-point is clinical disease, which may include nervousness, over-reactivity to external stimuli, incoordination, difficulty getting up and loss of weight. A scoring system will ensure that animals are culled when they reach clinical end-stage where clinical disease is highly suspicious of a TSE but the welfare of the animals is not severely

affected. For animals that need to be transported, fitness for transport will be confirmed prior to any transport.

For experimental infection, inoculation is performed through the skull into the brain under general anaesthesia, with possible adverse effects being haemorrhage, inflammation and cardiac or respiratory problems, potentially leading to death, although this has not happened in the past and measures are in place to prevent this from happening.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild for all naturally infected clinical suspects (all species) but moderate for the majority of exposed or experimentally infected animals regardless of species since they need to be kept until development of clinical disease unless exposed animals do not develop disease because of older age at exposure or genetic resistance (mild severity for these).

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Tests validation and quality control can only be achieved with tissues from the natural host.

The majority of animals are clinical field suspects or exposed on farms with known confirmed TSE cases, which are subject to cull as per legislation.

Experimentally infection is necessary to reproduce natural disease for those disease types where cases of natural disease are too rare or material is limited or unsuitable, for example due to autolysis.

Which non-animal alternatives did you consider for use in this project?

None. There are no suitable non-animal alternatives.

Why were they not suitable?

Cell cultures are not available to replicate prions from various species, and brain is needed for test validation and EQA purposes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on historical material demand and availability of naturally infected or exposed animals; the latter may be from farms that are subject to herd or flock cull as per legislation, which depends on farm size. As scrapie infection in sheep and goats is influenced by genetic predisposition (the prion protein genotype) and other factors, such as age at exposure, which is not usually known, the number of sheep and goats to be used is comparatively large but only a small number (less than 10%) in a flock or herd cull may be infected and able to provide scrapie-positive material.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Material requirements for test validation and EQA are discussed with the relevant stakeholders, including the biological archive manager, to estimate demand.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When possible, archived material will be used or material obtained from animals culled as part of an eradication measure of TSE as notifiable disease or found dead on farms and subsequently confirmed as TSE positive through active TSE monitoring. If experimental infection is required, the intracerebral instead of the oral route will be used to achieve a higher success rate of infection with shorter incubation period and thus reducing the number of animals needed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The natural host, i.e. ruminants, will be used. Most will be naturally infected and must be culled as per legislation, and experimental disease will only be generated for those disease types where naturally occurring cases will not provide adequate material. For experimental infection, intracerebral inoculations will be used as it most closely resembles naturally acquired spontaneous disease and reduces the time period that animals need to be housed indoors.

Why can't you use animals that are less sentient?

Tests have to be validated in the natural host.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All of the TSEs studied are naturally occurring and procedures to monitor animal clinically are well established. Clinical monitoring methods include regular clinical examinations and passive observations, including CCTV monitoring, in addition to daily observations during normal husbandry to determine onset of clinical disease and clinical end-point. Inoculations into the brain are carried out under general anaesthesia and analgesia with material that has been screened for microbial contamination prior to use to minimise post-operative adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Best practice guidance is obtained from NC3Rs, ARRIVE, IAT and the RSPCA. Publications and articles are also reviewed during the approval process prior to each individual study. Where specialist training is required, inter-institutional exchanges and training visits are organised.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The Establishment is a signatory to the Concordat on openness on animal research and applies the Culture of Care in animal studies as well as the ARRIVE guidelines. Staff from the Establishment frequently attend or organise external symposia on laboratory animal welfare e.g. RSPCA and IAT meetings. Staff attending these meetings provide meeting feedback reports locally. In addition, the Establishment has a Species Group Care and Use Committee where all personal licensees are invited to attend. Specialist topics are presented and refinements, such as environmental enrichment, are communicated and opportunities are used for implementation. In addition, specialist knowledge exchange is organised by field and lab exchanges with other research organisations.

122. Immunotherapy and vaccine development for cancer and sars-cov-2

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, vaccine, immunotherapy, SARS-CoV-2/COVID-19

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify and validate targets for immunotherapy, develop vaccines and immunotherapy strategies to treat melanoma, breast, lung, ovarian, pancreatic, colorectal and gastric cancers and SARS-CoV-2 and to progress at least two products to clinical testing.

To investigate the influence of microbiota on responses to vaccination/therapy.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer directly affects 1 in 2 of the population during their lifetimes and although significant progress has been made in the last two decades cancer still remains a major cause of mortality and improved therapies are required.

SARS-CoV-2 emerged as an international public health emergency and since first detection has led to 128,000 deaths in the UK. Emerging virus variants result in continued infections and fatalities. Vaccine treatments and therapies are showing promise although the efficacy of these against emerging variant strains remains unclear. It is therefore essential to develop new treatments to prevent and contain this virus. These will benefit the worldwide population to reduce the severity of this disease.

What outputs do you think you will see at the end of this project?

Development of novel immunotherapy products and combinations for cancer treatment and translation of these into clinical studies

Increase in understanding of the effects of immunotherapy on the immune system
Significant data publication in high quality peer reviewed journals

The translation of SARS-CoV-2 vaccine into clinical studies as a booster vaccine against emerging virus variants

Who or what will benefit from these outputs, and how?

In the shorter term, we expect to discover more about immune responses to cancer antigens and how best to harness these for cancer therapy. This will be of interest to both clinicians and research community interested in immunology and tumour immunobiology and will be made available through publication in peer reviewed journals.

The primary expected long term benefit will be the development of new therapies for treatment of melanoma, ovarian, lung, breast, colorectal, gastric and pancreatic cancers that are effective in causing tumour regression and prolonging patient survival.

The world wide population will benefit from SARS-CoV-2 vaccines that can be used as boosters to existing vaccines and help protect against emerging SARS-CoV-2 variants.

How will you look to maximise the outputs of this work?

Outputs will be maximised through ongoing and new collaboration with groups at research institutions, universities and biotech companies.

New knowledge will be formulated into scientific papers published in peer review journals and presented at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 13490
- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Currently rodent models are the most widely used models with physiological relevance to humans. Mice and rats aged over 6 weeks are chosen as models of mature immune systems and suitable for study of the interplay between the immune system and tumour growth. Mice genetically modified to express components of the human immune system enable better assessment of vaccines and immunotherapies for translation to human use. Immune deficient mice permit the assessment of immunotherapies using human tumour models or human immune cells in an in vivo setting.

Typically, what will be done to an animal used in your project?

Typical actions:

In this project, mice will be bred to have specific genetic modifications which are not expected to be harmful and will be bred using natural mating and maintained for use in this or other project licences.

The animals in this project may be injected multiple times with the candidate items that will be tested (by one or more of the following routes -subcutaneous, intravenous, intraperitoneal,

intradermal, intramuscular, gene gun, topical, drinking water, or diet) to assess whether the therapeutics are immunogenic.

Implantation of tumour cells subcutaneously or in the mammary fat pad will be performed and immunotherapies tested for prevention of tumour growth using pre-determined dosing strategies.

Luminescent imaging under anaesthesia may occur in some studies on typically up to 4 occasions.

Blood sampling, taken from a superficial vessel, will occur in some studies and an optional sample taken under terminal anaesthesia.

Animals may be transported to labs at the site of secondary availability for S1 killing and tissue harvest.

The typical duration of a study will vary from between 3-12 weeks and beyond, depending on the study design. Animals will be humanely killed at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

It is anticipated that some animals may experience minor transient discomfort during this project resulting from the implantation of tumour cells, administration of substances, in vivo imaging and blood withdrawal. During the tumour growth phase some animals may show signs of skin change over the tumour which will be monitored for up to 7 days with recovery. This will cause the animal moderate discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The techniques that may be used under this protocol are not expected to result in an animal experiencing clinical symptoms beyond mild to moderate transient discomfort.

Expected severities for mice in on breeding program is subthreshold. Mice on other protocols are expected to be 90% mild and 10% moderate.

Expected severities for rats are 97-98% mild and 2-3% moderate.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In vitro assays cannot adequately model the complete array of events involved in tumour growth, or the generation of an immune response, both of which are essential to our understanding of tumour immunology and anti-viral responses. In vivo studies using rodent models are therefore crucial.

Which non-animal alternatives did you consider for use in this project?

Whenever possible, a variety of in silico and in vitro experimental techniques to identify potential immunogenic peptides/proteins will be used to minimise animal use prior to in vivo studies.

Why were they not suitable?

The in silico and in vitro models fail to truly represent the complexity of the immune system and the establishment of immune responses. In vitro tumour culture fails to truly mimic the

in vivo growth of a tumour and does not efficiently replicate the complex tumour environment that is established in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals have been estimated based on previous usage and expected usage based on progress using our experimental therapies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Studies will be designed to use the minimum number of animals required for statistical significance.

We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculation studies. We will consult qualified statisticians about experimental design and statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All experiments using live animals will adhere to the ARRIVE guidelines on design and reporting. Good principles of experimental design will be applied to ensure sufficient group sizes will be used to adequately test the hypothesis. Sample sizes are estimated from pilot studies and previous data using power analysis

Breeding will be performed to reduce any surplus mice and both sexes will be used in studies. Consideration may be given to the use of ex-breeders for the use of tissue collection and analysis.

Candidate vaccines will be designed and assessed for their suitability with the use of in silico and in vitro techniques before the possibility of moving into an animal setting.

Mice with multiple rather than single relevant genetic modifications will be used whenever possible.

Tumour cell lines will be maintained in vitro and stored frozen to negate the need to use additional animals for maintenance in vivo.

Pilot tumour growth studies will be performed where appropriate to establish tumour growth prior to use in therapy studies.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice of a non harmful phenotype will be bred and maintained.

All cells prior to implantation are prepared in a sterile environment and are administered using sterile techniques and the lowest volume of cells.

Substances are prepared and administered using sterile technique. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be in accordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

The assessment of tumour development will be performed using an appropriate measuring device.

The delivery of some therapies will be assessed using the in vivo imager which will require transient anaesthesia to immobilise them while an image is acquired. Once completed, the animals are expected to make a full recovery within 30 minutes.

Blood sampling will be performed using sterile techniques and the volumes collected will be in accordance with the LASA / NC3Rs guidelines. We will aim to take the smallest volume which will allow for adequate analysis.

All animals will be humanely killed by a Schedule 1 method.

Why can’t you use animals that are less sentient?

To efficiently assess the proposed project animals need to have a fully functional mature immune system. Animals younger than 6weeks old or at an early gestational stage of life are not sufficient to model a fully functional immune system. Mice and rats are the lowest sentient organisms appropriate to use to model the interplay of the immune system and tumour development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The route, volume and frequency of the administration of substances is reviewed and selected in order to ensure the scientific question can be answered with the least impact on the welfare of the animal.

Where a procedure has an unexpected impact on the welfare of animal the frequency of monitoring clinical signs, bodyweight and/or tumour development will be increased.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by the Workman Group on welfare and use of animals in cancer research published in 2010, The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and LASA Good Practice Guidelines.

I will also take note of publications relating to improving the reproducibility of animal research such as Frommlet, F. Improving reproducibility in animal research. Sci Rep 10, 19239 (2020). https://doi.org/10.1038/s41598-020-76398-3.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain close interactions with the NACWO, NTCO and NIO as they oversee and perform in vivo studies. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and the newsletters which are circulated monthly by the NIO.

Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

123. Studying host interactions in tumour and metastasis

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, microenvironment, metastasis, immune cells, inflammation

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This Project licence aims to support the work of the laboratory in performing mechanistic studies on the interaction between tumour cells and the healthy host tissue cells. We have a particular interest in the relationship between tumour and inflammation and its relation with the rest of the cellular environment of cancer cells.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One out of two people will get cancer in their lifetime, making cancer one of the most common diseases. Over the last 40 years, cancer survival has doubled largely thanks to better treatments but despite this more than 1 in 4 deaths in the UK is still caused by cancer. The fundamental understanding of cancer complexity is instrumental for the development of new and refined therapies. The studies on the synergistic cooperation

between tumour cells and the host tissue cells holds the potential for novel and more effective anti-cancer therapies. Indeed, the interaction of cancer cells with their microenvironment is implicated in every aspect of tumourigenesis, from onset to metastasis as well as in resistance to chemotherapy. Understanding the inflammatory component of the microenvironment is of particular interest for the enhancement of efficacy of host-dependent anti-cancer treatment like immunotherapy.

However, this complex physiology needs to be addressed within an mammalian system. Mice have a comparable physiology and the tumour disease trigger the same cascade of events as in humans. In particular, the human immune-system's reactions to cancer are highly recapitulated in mice, which are fundamental part of any therapeutic intervention and an essential focus of our work.

What outputs do you think you will see at the end of this project?

The expected benefits of the work will be mechanistic understanding in the immediate future and therapeutic benefit in the longer period. They can be summarized as follows:

1. To assist in the design of future anti-cancer therapies. We aim to discover novel molecules and proteins involved in the tumour microenvironment that can be efficiently targeted to weaken tumours making them more sensitive to standard therapies and reduce the risks of recurrence.

2. We aim to understand the impact of environment and lifestyle (for example; diet, inflammatory reaction, microbiota) on the tumour microenvironment and the impact on spread (metastasis) and cancer cell reactivation (dormancy). This will help the management of cancer patients and potentially help identify patients at higher risk of recurrence.

3. By studying the involvement of certain inflammatory components and systemic signals in cancer, we aim to clarify the mechanism of disseminated dormant cancer cell re-activation.

4. We also aim to better understand novel relation between cells of the tumour microenvironment and particularly the relation between the nervous system, inflammation, and cancer initiation.

5. Finally, this work will benefit the basic research community by increasing our knowledge of tumour biology.

Who or what will benefit from these outputs, and how?

The output of this work will initially be mechanistic information on cancer progression, identifying new potential drug targets, which will be used by the pharmaceutical industry to guide future potential treatments. The work will also provide valuable information to guide

doctors in the clinic for the management of cancer patients with disease that may have spread to other sites in the body.

The short-term benefit will include presenting at scientific conferences and supporting other in vitro findings followed up with the publication of novel discoveries in international scientific journals. In the mid and long term the benefit will reach the pharmaceutical industry and clinicians, resulting in improvement of care for patients.

How will you look to maximise the outputs of this work?

We will look to maximise the outputs of any significant findings through interactions with the scientific community through presenting at seminars and conferences. We also aim, where possible, to present unsuccessful data to prevent repetition by other labs. We also work alongside the Translation team who support collaborations with industrial and clinical partners that would reduce the bench-to-bedside time and enable possible changes in clinical practice (use of new drugs/screening tools) to be trialled sooner.

Species and numbers of animals expected to be used

- Mice: 45000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

My area of research is cancer; therefore, mice are for me the species of choice.

The reasons why mice are the best choice as cancer experimental models, can be summarized as follows:

- the physiology of cancer in mice is consistent with the human disease,

- The genome of a mouse is easy to modify allowing the study of particular genes in the cancer process. Mice can also be modified to be genetically prone to cancer (spontaneous genetically engineered mouse models) and this can allow a less experimentally engineered approach to studying the development and spread of cancer.

- In mice, there are many models that are commercially available, as well as well-defined techniques for production. There are also many scientific tools like genomic libraries, antibodies etc that are largely available.•

In most cases where tumours will be induced, adult mice will be used, but there will be instances where juvenile animals may be used, for example; in order to pre-condition with dietary changes.

Typically, what will be done to an animal used in your project?

Typically, animals will develop tumours (either spontaneously through genetic modification or through transplantation of tumour cells by injection methods). In some cases, we will transplant tracer cancer cells (created by our lab) that can label the cells neighbouring the tumour, therefore tissue will be collected to allow further analysis.

Treatments (chemo/radiotherapy) could be employed in this setting. Drugs or antibodies will be used to study the roles of different host cell types on the tumour disease, generally by depleting or boosting them and looking at how the tumour develops, spreads and responds to treatments such as chemo or radiotherapy.

As we are interested in how environmental changes also impact cancer progression, we may use other interventions, such as; changes to the diet, infections with respiratory viruses, changes in gut bacteria (using antibiotic treatments). These interventions will have impacts on the host and we are interested as to how the changes caused by these will impact on the tumour and it's response to therapy.

The animals might have their disease monitored over time using non-invasive imaging techniques, particularly if they have internal disease. The duration of the experiments will vary depending on the model and will consider many features of the disease specific to the experiment, including the nature of the cancer cells, if transplanted (slow or fast growing, local disease or prone to spread) or the known timeframes in genetically engineered mice that form spontaneous tumours. We always aim to maintain duration of experiments to the minimum required to address the scientific need.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases animals used in this license will form tumours. The size and impact of these tumours on the animal will differ due to model and method. For example, poorly aggressive tumour cells might grow well within the transplantation site but spreading to other areas of the body is poor and therefore the impact on the animal is low. However, with a more aggressive line tumours may spread early meaning that even though growth at the transplant site is not particularly high, the impact on the animal due to spreading to other areas of the body may produce more suffering for the animal.

We have much knowledge and experience of all the models that we use, both transplantation and spontaneous and are able to predict well the time frame over which the animals will not show adverse effects. The use of certain interventions can make this slightly less predictable, but the animals are monitored closely in this case to minimise any suffering and we aim to terminate experiments before the onset of undesirable adverse effects. Undesirable affects could be limited to the locality of the tumour (for example- ulceration) or be general signs of poor health, such as; weight loss, piloerection, hunching, increase breathing rate (especially when tumours have spread to the lungs).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities predicted by this proposal are both mild and moderate.

For those protocols that involve tumour growth it is expected that a greater proportion (50%) will reach a moderate severity, due to repeated procedures, tumour burden or possible surgery.

When working with animal models it is essential to minimise any possible adverse effects of the experimental procedure. We closely monitor the animal's reaction to specific experimental procedures and pay attention to any sign of suffering that may occur. Cancer is a complex disease, however the knowledge of all the tumour models used will allow a good prediction of the time frame of the disease progression. Therefore, mice will be likely killed on the basis of a time period rather than based on their clinical signs. An experimental end point (time controlled) will most likely occur before a humane endpoint (as determined by deterioration of heath conditions) and result in only mild suffering for the animal.

When performing our metastatic studies, it will not be an experimental requirement for animals to reach a late stage of disease, but metastatic progression must occur. We will pay particular attention to mouse behaviour and monitoring specific to the organ targeted by the disease, and will in most cases, aim to terminate experiments before obvious signs of health deterioration appear. For example, in cases where tumours spread to the lungs, particular attention will be paid to the breathing behaviour of the mice both in resting condition and after a small physical challenge, however, some mice can drop in condition very quickly without many prior signs and may show more of a moderate suffering.

What will happen to animals at the end of this project?

- Killed
- Used in other projects
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In contrast to other tumour studies, here, a tumour is analysed in the context of its local tissue environment, as well as within the scope of the systemic changes induced in the

host organism affected by the disease. Therefore, these studies must mainly be performed within the animal as the complexity of these changes and the number of players involved cannot be modelled in the laboratory.

Which non-animal alternatives did you consider for use in this project?

Targeted lab-based assays using 3D scaffolds and organoid cultures will be designed accordingly with the gained information used to reduce mouse workload. For instance, in order to assess specific interactions between tumour cells and certain components of the microenvironment or of the immune system. This approach will also have the advantage of identifying the impact of these components on each other in a “clean” system where all the players are known and better controlled.

Why were they not suitable?

Even the most sophisticated lab-based model systems cannot convey the complexity of the tumour microenvironment or the impact that tumour disease can have systemically on an animal. In order to investigate the impact of infiltrating cell types alongside both the local and systemic response of tumour we can only do this within the complexity of the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used have been estimated on the basis of the previous 10 years of work of my lab and the current landscape of projects of the lab going forward.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We employ several strategies to try to limit the number of mice in the study:

- Firstly, we will always aim to maximise the amount of data we get from each mouse and when possible, we will use it for the study of both primary tumour and metastasis.
- Also, we will limit the use of genetic models (that often require many generations breeding) using orthotopic transplants of labelled cells and treating the mice with chemical agents either to block immune-system components or to generate tumours.

- We also use the minimal number of mice needed for statistical significance when testing the experimental hypothesis based upon pilot experiments to inform on numbers required. We can always relay on our in house statistician for any additional advise whenever we need.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Whenever possible, we will share animal tissue from experiments to enable multiple studies-ex vivo.

We are committed to improving education and training for those working under this project license.

We breed many of the genetically altered animals ourselves in order to promptly and, often transitory, adapt colony sizes to respond to the experimental need and reduce wastage from overbreeding.

We also take lead from HO efficient breeding of GA animals (https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/773 553/GAA_Framework_Oct_18.pdf) and take decisions to archive lines by cryopreservation when not required over a period of time.

Obtaining wildtype mice from in house facility-shared breeding allows better efficiency for larger colonies.

We we also always run pilot study to estimate the effect size and the directions for future experimental settings.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use spontaneous genetically engineered models of cancer alongside more controlled experimental models of inducing cancer, including transplantation techniques. Our work at present is mainly focussed on breast cancer and pancreatic cancer but it is not limited to this. We minimise the use of surgical methods, wherever possible, by

employing ultrasound guided injection techniques which drastically reduces the suffering experiences by the animals.

Why can't you use animals that are less sentient?

Other animals that are suitable for these types of studies lack the organ and physiological complexity needed for translation to humans. This complexity needs to be recapitulated to make the investigation of tumour microenvironment possible. The requirement here is to have a physiology which is as close as possible to humans, and only mammalian organism have the same complex immune system, hormonal infrastructure and basic metabolism as humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have access to cutting edge techniques and experts within various fields of medical research. We actively share refinement and improvements in techniques and seek to constantly improve our models to ensure that we are minimising any harms to the animals, as this also helps to improve the accuracy of our study and reduce artefacts caused by stress. For example; the use of imaging guided techniques to reduce surgery as a means of transplanting cancer cells to internal sites. We also follow local NVS policy on post-operative care and pain management to minimise any harms to the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Unless otherwise specified, the work in this project will be designed using the principle outlined in PREPARE guidelines for planning animal research and testing (2017) and in the LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery.

With regards to cancer models we take advice from the "Guidelines for the welfare and use of animals in cancer research".(Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford IJ, Tozer GM, Watson S, Wedge SR, Eccles SA; Committee of the National Cancer Research Institute. Guidelines for the welfare and use of animals in cancer research. Br J Cancer. 2010 May 25;102(11):1555-77. doi: 10.1038/sj.bjc.6605642. PMID: 20502460)

LASA Guidelines will be followed for Administration of substances.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly receive updates on advances in the 3Rs from within our establishment from NC3Rs and NORECOPA. Where we are placed to refine techniques without impacting the scientific validity of our work we aim to implement advances.

124. Developing treatments for huntington's disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Huntington's disease, Neurodegeneration, Therapy

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This projects aims (1) to understand the first steps in the process by which the faulty gene in Huntington's disease causes brain cells to become dysfunctional and die and (2) to test treatments designed to correct these early stages of the disease process. It is by targeting these first steps that we might develop the most effective medicines to delay or slow down the course of the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

HD is a devastating disease of the nervous system. On average, symptoms start at around 40 years of age, but this can vary widely from early childhood to very old age. Affected individuals develop psychiatric illnesses, an inability to control their movements, difficulty in

reasoning and solving problems and lose weight. In the latter stages, HD patients are often bedridden and unable to talk or swallow (being fed via a feeding tube). The disease progresses for 15-20 years until death. Children of affected individuals have a 50% chance of inheriting the faulty gene and have often witnessed the effects of this devastating disease in their relatives. There are no treatments that will delay or slow down the course of the disease and most of the symptoms do not have effective medicines.

What outputs do you think you will see at the end of this project?

1. An in depth understanding of which of the products of the faulty Huntington's disease (HD) gene are the most damaging to brain cells.

2. A detailed characterization of the effects of the faulty HD gene in the brains of HD mouse models.

3. A understanding of which treatments are most successful at decreasing the damaging HD gene products and have the most beneficial effects.

Who or what will benefit from these outputs, and how?

The Huntington's disease research community will benefit. We will gain a better understanding of how the faulty HD gene causes brain cells to become dysfunctional. Other researchers will build on this information to further our collective understanding of the disease. These benefits will occur during the course of this project.

Biotechnology companies and pharmaceutical companies will benefit. In order to develop treatments for Huntington's disease, researchers in universities and in industry must work together. Our research into the cause of the disease will help industry decide on which are the best treatment options to follow. We shall use our very detailed knowledge of presymptomatic disease in HD mouse models to collaborate with colleagues from academia, industry and he HD Foundations to test potential therapies in mice. This will help companies determine which are the best treatments to develop as medicines that can be given to people. These benefits will occur during the course of this project.

Huntington's disease patients and families will benefit. We shall target the first stages by which the faulty HD gene causes brain cells to dysfunction. This has the potential to produce the most effective medicines to delay or slow down the disease process. Our collaborative research program will help to decide which are the best approaches to test in clinical trials. The tolerability of such treatments could be tested in HD patients during the course of this project and large-scale clinical trials could be initiated.

How will you look to maximise the outputs of this work?

To accelerate progress, we shall work with collaborative networks of scientists in academia and industry that are coordinated through Huntington's Disease Charitable Foundations. We will share results informally and formally at workshops and conferences.

We will publish completed projects in open access journals (that are free for anyone to read) and share them before publication on an appropriate website.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We cannot learn about the early stages of Huntington's disease in brain cells by studying HD patient tissue. We only have access to this as post-mortem brain by which time so many things have gone wrong that we cannot unravel the first steps in the disease process. We need to target these first steps to develop the most effective medicines.

Mice have a nervous system of comparable complexity to humans and we can use them to study degenerative conditions of the brain. We know that mouse models of Huntington's disease are good models of the human disease. Over the past 25 years, we have learnt about many important aspects of the disease, from studying the mouse models, of which we were previously unaware. HD is a late-onset disease and therefore, we shall usually work with adult mice, although we may also use younger mice to study the first changes in the brain.

Typically, what will be done to an animal used in your project?

We shall use many of the animals in this project for breeding.

We shall breed HD mouse models to provide tissues and brain sections to map out changes in the brain before symptoms begin and during the course of the disease.

We shall breed HD mouse models to other genetically altered mice (which do not themselves have symptoms). This will be to ask whether these genetic alterations delay or slow down the presymptomatic brain changes, or symptoms of the disease.

We shall treat HD mice with potential medicines to find out whether they delay or slow down the presymptomatic brain changes, or symptoms of the disease.

We may be able to give these treatments in the food or drinking water. However, some of the treatments that are being developed for HD need to be given directly to the brain. In patients, these therapies are delivered to the fluid that surrounds the brain (CSF) by a lumbar puncture or into the brain tissue. In mice, they are delivered into the CSF in a space in the brain by injection. Therefore, some mice will undergo surgery under anesthesia.

In most cases, we will collect tissues to study brain cells before symptoms begin. In some cases, we will monitor symptoms with tests to measure e.g. weight, changes in gait, balance and coordination, anxiety, memory and grip strength. These are short non-invasive tests that are repeated at appropriate intervals. Gait measurements may take a few minutes, monitoring exploratory activity may take 30 minutes.

At the end of a study, we will collect tissues for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

HD mouse models develop symptoms that are similar to those seen in the human disease and include changes in gait, problems with thinking and a failure to gain weight.

Most of the HD mouse models that we use do not develop these symptoms until after one year of age and we do most of our work before they develop symptoms. We sometimes keep mice after symptoms appear. One of our mouse models develops symptoms at a younger age and we may use this model to find out how much a treatment improves symptoms.

Some of the treatments that are being developed for HD require administration into the fluid that surrounds the brain. Therefore mice receiving these treatments will require surgery under anesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The level of severity associated with the symptoms of Huntington's disease is moderate. However, the vast majority of our work is performed using mice that are in the presymptomatic phase of the disease. Some of the treatments that we shall test need to be administered directly into the brain under anaesthesia. We anticipate that approximately 20% of our mice will have a moderate level of severity.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to study the earliest stages of the disease in order to develop effective medicines for Huntington's disease. As HD is mainly a disease of the brain, patient tissue is not available for this purpose.

Our work is part of a large international research program. The processes that we study often follow on from work in cultured cells or invertebrate HD models (flies and worms). However, in the past, we have tested many potential treatments that have been suggested by work with these simple systems and have usually obtained negative results in our HD mice.

Clinical trials for HD are extremely expensive. This is because several hundred patients must be studied over a period of two to three years to give a good chance of detecting a beneficial effect. Therefore, potential treatments suggested by work with the simple systems cannot be tested directly in clinical trials.

We use HD mouse models to provide an important intermediary resource to test potential therapies and prioritize those that should be advanced for clinical trials.

Which non-animal alternatives did you consider for use in this project?

The only possible non-animal alternatives might be HD brain cells that have been made by reprogramming the skin or blood cells from an HD patient to generate stem cells. Over a period of a few months, these stem cells can be turned into brain cells in a dish.

Why were they not suitable?

Huntington's disease is a slowly progressing brain disease with an average age of onset of 40 years. Brain cells that have been generated from stem cells and grown in a dish cannot model the course of the disease. At present, these cell-based systems cannot reproduce the complexity of the mammalian brain.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Our experimental design is based upon on extensive knowledge of HD mouse models. Through our experience in planning these studies and consultation with statistical experts we use the minimum number of animals required to obtain scientifically valid and robust results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1. We are aware of the minimum number of animals required to obtain valid results in our tests. We design our studies to limit the number of experiments required.

2. We have developed tests that can be performed on tissues at early stages of disease. Therefore younger mice that have not developed symptoms can be used. These tests require less mice than those that measure neurological symptoms in symptomatic mice.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. We have developed approaches that could, in some cases, replace complex breeding programs thereby reducing the number of mice required.

2. We have found that one of the products of the HD gene, that initiates the disease process, is produced in skin cells from HD mice. We have immortalized skin cells from our mouse models to provide a continuous supply of this resource. We are using these to investigate the disease process and for screening potential therapeutics. The most suitable therapeutic can then be tested in mice to study their effect on the disease process in the brain.

3. Breeding mice can produce more animals than are needed. We take tissues from these additional mice and maintain an HD model tissue bank. This means that we often have a ready source of tissues available for analysis without the need to breed mice. We can also provide the HD scientific community with a tissue resource, removing the need for some labs to keep HD mice.

4. We freeze embryos or sperm for all of our mouse lines, so that we do not need to breed live mice unnecessarily.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We shall use mouse models of Huntington's disease. We shall age mice to provide tissues for biochemical or histological techniques. In most cases the tissues will be collected before the mice show symptoms of the disease. Sometimes, we will monitor the mice by observational tests or tests of strength, gait or memory. These are non-invasive and we will halt the test if a mouse shows signs of fatigue. In other cases, we will study the brains of the mice by imaging techniques under anaesthesia, from which mice will make a full recovery. Mice will be tested with potential medicines to see if they have beneficial effects.

Why can’t you use animals that are less sentient?

We are studying the earliest stages of the process that causes brain cells to become dysfunctional in Huntington's disease. To do this, we are studying the way in which the HD mutation alters the processing of the HD gene and its products. The structure of the HD gene is conserved between human and mouse and so we can use HD mouse models for this purpose. The structure of the HD gene is not the same in other possible animal models e.g. flies and worms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have adopted best practice with respect to housing e.g. the use of environmental enrichment, and of cylinders to pick up mice. In most cases we study our mouse models of HD before they develop symptoms, by performing tests on tissue samples. These tests need less mice to give a conclusive result than the tests that monitor symptoms e.g. changes in gait. If we do need to measure clinical symptoms, we do not use tests that would cause pain or distress. We monitor symptomatic HD mice with a scoring system to ensure that they always have access to food and water and never become immobile or unresponsive. We will review and update these procedures following guidance on the NC3Rs Resource Hub.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will access the experimental design resources available through the NC3Rs Resource Hub. This provides access to a wealth of guidelines e.g. ARRIVE and information on the design and analysis of experiments at all stages.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our NC3Rs representative is a member of our Biomedical Services Users group and will keep us informed of advances at these meetings. We shall use the NC3Rs Resource Hub to keep up with best practice. We subscribe to the NC3Rs newsletter and will access NC3R E-learning modules and webinars.

125. Mouse models of chronic inflammatory clinical conditions. Word document missing

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

inflammation, immunology, immunotherapy

Animal types	Life stages
Mice	neonate, adult, aged, pregnant, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify pathways that contribute to chronic inflammatory conditions that underly clinical diseases afflicting many people and to use this information to develop and validate new treatments to prevent, control or cure these conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Chronic inflammatory conditions are clinical diseases that reduce patient quality of life slowly as disease symptoms become worse. These conditions include persistent infections, cancer, autoimmune, allergic, neuroinflammatory and cardio-vascular diseases, as well as rejection of transplanted cells, tissues and organs. Many people, particularly the elderly, succumb to one or more of these conditions. For example, in the UK, 85% of people over the age of 85 currently suffer from more than one chronic condition. Comorbidities complicate treatment options and medical management for many elderly people but chronic conditions such as cancers, chronic infections and autoimmune syndromes also afflict many younger people. Despite the diversity of these clinical conditions, sustained tissue inflammation is a common underlying factor. The purpose of inflammation is to activate immune responses but when sustained inflammation may cause tissue injury and symptoms that get worse over time, including pain, fatigue and behavioural disorders. Collectively, chronic clinical conditions present a huge and growing burden on health care systems worldwide as disease incidence and severity increase with age, complete cure is rare, and relapse is common. As mice and humans are mammals their immune systems share many common features, and mouse models of chronic clinical conditions replicate clinical conditions experienced by patients. Hence, mouse models offer powerful ways to gain new insights into physiologic processes driving chronic inflammatory disease progression and resistance to therapy, and to develop and test new immunotherapies to treat these debilitating clinical conditions.

What outputs do you think you will see at the end of this project?

Outputs from this project will include new information about underlying physiologic processes that incite and sustain chronic inflammatory conditions. Information generated will identify potential new targets to prevent, control or cure these conditions by manipulating inflammatory and immune responses (immunotherapy) for clinical benefit. New reagents showing therapeutic promise in mouse models of chronic conditions will be developed for future clinical applications in partnership with clinicians and corporate partners. Outputs will also include publications and scholarly reviews in scientific journals, as well as presentations at scientific meetings nationally and internationally. Outputs will also include engagement with the wider public, healthcare professionals, patients and patient support groups, and charitable foundations supporting biomedical research on human diseases.

Who or what will benefit from these outputs, and how?

Over the 5 year period of the project, short-term project outputs will benefit the scientific and medical communities interested in preventing or treating chronic inflammatory conditions. Outputs will be communicated to these beneficiaries via publications, meeting

presentations and research seminars. In the longer term (5-10 years) expected project benefits are development and validation of new treatments to prevent, control or cure chronic inflammatory conditions in partnership with other scientists, clinicians and corporate partners.

How will you look to maximise the outputs of this work?

Outputs will be maximised by publishing project data in a timely fashion in scientific journals and scholarly reviews, and by presenting research talks at meetings and seminars. Collaborations with other scientists, clinicians and corporate partners will be sought to enhance project scope, quality and productivity. New knowledge dissemination will include unsuccessful, as well as successful outputs to ensure that new insights into disease processes generated from the use of mouse models of chronic inflammatory conditions are distributed effectively and efficiently.

Species and numbers of animals expected to be used

- Mice: 15,300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As mammalian species, mice and humans share multiple features arising from a long period of co- evolution, including an immune system composed of many common cell types and functions. In addition, mice breed relatively quickly, can be bred to generate new strains with defined genetic backgrounds, and mouse genes can be manipulated to create new strains used to study disease mechanisms. Consequently, over many decades multiple mouse models of human disease have been developed and used to study clinical disease processes. To date, mouse models have generated key insights into physiologic processes driving many clinical diseases, including (but not limited to) cancers, infections, allergies, autoimmune syndromes and transplant rejection. In this project, adult and aged adult mice will be used to study how chronic inflammatory conditions arise and develop in mice with mature immune systems, and to study how age impacts these disease processes and treatments.

Typically, what will be done to an animal used in your project?

The project uses several different mouse models of chronic inflammatory conditions. The overall project rationale is that sustained tissue inflammation is a common physiologic process driving chronic activation of the immune system, leading to disease progression and common co-morbidities experienced by patients with such clinical conditions. Some

mice will be bred in house (under SPF conditions) to generate standard and GA strains needed for experimental research (Protocols 1, 2).

Protocol 3 allows healthy adult mice to be treated with reagents that stimulate or suppress inflammatory and/or immune responses (Protocol 3). Sustained inflammatory and immune responses may incite disease progression and onset of clinical symptoms specific to each disease such as persistent (Protocol 4) infection, tumour growth (Protocols 5,6), or immune-mediated tissue injury and gradual loss of tissue function (Protocol 7). As the current Covid-19 pandemic has shown, pre-existing chronic inflammatory conditions such as chronic infections may increase risk of potentially lethal respiratory infections. To address this important clinical complication, this project includes a comorbidity model to study how established chronic infection impacts host immune responses to treatments for cancer (Protocol 4).

Experimental duration depends on the particular disease model under scrutiny. Mice infected with murine retrovirus (MuLV) develop persistent infections and may be kept for up to 6 months (Protocol 4). Most mice with transplantable tumours are kept for up to 2 months after engrafting tumour cells (Protocols 5). Some mice that clear primary tumour burdens following effective therapy, and mice with prior MuLV infections may be kept for longer (up to 9 months) to allow research questions to be fully addressed; for example, mice may be re-challenged with tumour cells to assess if anti-tumour immunity induced after primary challenge and therapy is stable and durable. Mice prone to developing intestinal (colon) tumours (APCmin/+ mice) may be kept for up to 15 weeks (Protocol 6). Most mice used to study arthritis (Protocol 7) are kept for 4-6 weeks after initiating disease, unless therapies are effective when mice may be kept for up to 6 months to assess therapy efficacy.

Several procedures may be applied to mice, including (a) treatments to initiate disease, which may include booster treatments, (b) treatments to manipulate inflammatory or immune response pathways (given before, during or after disease initiation) and (c) potential new immunotherapies to prevent control or cure disease (given after disease initiation, at disease onset or when disease peaks). In some cases treatments or therapies may be applied daily over periods of up to 20 days.

What are the expected impacts and/or adverse effects for the animals during your project?

Expected impacts and clinical symptoms differ according to the disease model under scrutiny as follows;

Protocols 1 & 2: GA mice with harmless (Protocol 1) or harmful (Protocol 2) effects are bred locally to generate mice used for experimental work in other Protocols.

Protocol 3: Treatments to incite, inhibit or manipulate inflammatory and/or immune responses may cause transient mild discomfort that resolves in 1-2 days in most cases. Most reagents have been used for decades (e.g. Complete Freund's Adjuvant, CFA) and

adverse effects are well documented, though some novel reagents (e.g. hydrogels, DNA nanoparticles) may induce unanticipated adverse effects.

Our group is highly experienced in monitoring mice treated with such reagents to detect any unanticipated adverse effects and minimise them, as described under '3Rs'.

Protocol 4: Persistent MuLV infections cause increasing immune dysfunction manifesting as progressive splenomegaly, lymphadenopathy and immune suppression by 6-8 weeks post infection (99% incidence in susceptible strains). Infected mice are monitored regularly to assess general health and condition (activity, behaviour, appearance, body weight). In rare cases (<5%) swollen lymph nodes cause breathing difficulties, necessitating humane killing.

Protocols 5: Transplantable dermal or lung tumours are not expected to cause more than mild discomfort or interfere with the ability to access food and water or incite abnormal behaviours for several days (typically 7-12 days) after tumour cell transfer. Over time, increasing tumour burdens may cause general deterioration in health status, unless effective therapy is applied to slow tumour growth. Treatments that modulate inflammatory and/or immune responses may lead to stable control of tumour growth and some treatments may promote long-term survival (curative therapy). Some untreated dermal tumours may cause reddening or scabbing of skin (up to 10%), and some mice may develop skin ulceration (up to 1% for untreated mice and higher for some treatments).

Protocol 6: Most APCmin/+ mice (>90%) develop intestinal (colon) neoplasias causing moderate discomfort due to progressive loss of colon function and morbidities caused by loss of digestive functions by age 4-5 months, as documented in published studies using this model. Humane endpoints are set at age 15 weeks in this project to ensure that mice do not experience severe discomfort.

Protocol 7: Most mice (>70%) treated to induce inflammatory arthritis develop limb joint swelling that leads to increasing lameness (from mild to moderate) and local pain as disease progresses. These adverse effects do not impair access to food and water or ability to nest and interact with other mice. In monoarthritis models (induced by local challenge), most mice (>70%) develop swollen joints after ~24- 48h, which then declines over a 14d period (100%). Sustained inflammation may lead to skin ulceration in some WT mice (<10%) and incidence may be higher in some GA strains with defective immune regulatory pathways. Systemic polyarthritis models are as for local monoarthritis models, exept that all limb joints may become inflamed and joint swelling is progressive if no treatment is applied (50-100%).

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

Expected maximum severities and proportions (%) of animals in each severity category are as follows; Protocol 1: sub-threshold (~50%) or mild (~50%)

Protocol 2: mild (~80%) or moderate (~20%) Protocol 3: mild (~90%) or moderate (~10%) Protocol 4-7: mild (~5%) or moderate (~95%)

Note: Humane endpoints defined in each protocol ensure that severity and incidence do not exceed the proportions stated above.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Underlying physiologic processes that cause and sustain chronic inflammatory conditions are poorly defined, largely because disease progression (a) occurs long before clinical presentation and (b) is driven by interactions between small numbers of distinct cell types, many of which cannot be cultured ex vivo. Studies in mice preserve tissue architecture and physiologic complexity, allowing the effects of defined genetic or physical manipulations on complex responses to be evaluated over time starting from tissue homeostasis. New insights into disease processes and drugs targeting such processes identified using alternative approaches must be validated in vivo because the complexity of the immune system cannot be replicated ex vivo and the potential for misleading and potentially fatal responses to interventions is therefore high. These considerations mean that it is impossible to discern critical pathways that cause or prevent disease without recourse to in vivo disease models. Hence, studies on mice are essential to discover new drug targets for immunotherapy and validate new reagents as potential immunotherapeutic drugs to treat disease. Immunotherapy offers novel ways to improve treatments for syndromes that afflict many people, including those addressed in this project; cancer, infectious and autoimmune diseases. These opportunities arise from novel insights generated from fundamental research using animal models to identify physiologic pathways that initiate sustain, inhibit disease progression or alleviate established disease. For example, our past research using animal models of pregnancy and cancer identified the metabolic enzyme indoleamine 2,3 dioxygenase (IDO) as a process that protects developing foetal or malignant tissues due to suppression of maternal or anti- tumour immunity, respectively. Elevated IDO activity also manifests in other chronic inflammatory conditions because IDO is induced by interferons made in inflamed tissues. By analogy to cancer, elevated IDO activity may protect infected tissues from host immunity to promote pathogen persistence. On the other hand, interventions to boost IDO activity may protect healthy tissue subjected to immune- mediated injury in autoimmune diseases or transplant rejection.

Which non-animal alternatives did you consider for use in this project?

Alternative approaches considered include studies on;

(a) immortalized human or mouse cell lines,

(b) primary hematopoietic (bone marrow) cells

(c) studies on cells or tissues from healthy human subjects or patients.

Such approaches can generate mechanistic insights into underlying molecular pathways that contribute to disease or therapeutic responses.

Why were they not suitable?

The alternative non-animal based approaches considered exclude potentially critical contributions from other cell types (e.g. immune cells in cancer development and therapy) and distort, oversimplify and therefore devalue data generated. Historically, it has proven difficult to model complex physiologic processes that regulate (suppress) immunity using non-animal based approaches, largely (but not exclusively) because (a) small numbers of immune regulatory cells may control much larger cohorts of immune effector cells and (b) the difficulty of culturing immune regulatory cells, while preserving their regulatory functions and measuring regulatory functions ex vivo. Moreover, samples from patients provide few hints about underlying causes of inflammatory diseases that develop over many years because it is not possible to access tissues where early disease development is initiated and progresses long before clinical presentation. A further complication is that identical ligands and signal pathways may incite opposing effects (immunogenic or tolerogenic) in distinct physiologic cell types.

For example, inflammatory insults co-activate parallel pathways that stimulate and suppress immunity, and the balance between opposing responses is elaborated in physiologic systems to incite or inhibit disease processes or therapeutic responses. Without recourse to in vivo disease models to verify particular outcomes there is substantial danger that potential new reagents to treat disease may instead exacerbate disease in clinical settings. In summary, in vitro approaches do not replicate the complexities of tissue microenvironments, and data generated in vitro must be validated for relevance in vivo due to the high risk that extrapolations from alternative (in vitro) approaches may result in misleading (at best) or fatal (at worst) responses in vivo.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of mice required for this project were estimated based on the following criteria:

1. Prospects to fund specific avenues of research on particular models of clinical disease described in this application.

2. The need to breed standard mouse strains and GA strains in house to generate mice required for this project (up to 7,500 GA mice in Protocols 1 & 2 over 5 years).

3. Experimental group size necessary to test scientific hypotheses rigorously.

4. Control groups required to test scientific hypotheses rigorously.

5. Replicate experiments (at least 2) necessary to validate study outcomes and minimise variables in order to test scientific hypotheses rigorously.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Measures to minimise numbers of mice required for this project include the use of well-defined interventions and parameters (readouts) appropriate to the research questions posed. Studies will involve the minimum number of mice per experimental group and experimental replicates (2) to generate unequivocal outcomes. Measures to minimise numbers of mice required for this project include use of the following guiding principles;

1. Using standard interventions developed over many years, and/or used in many laboratories, that induce well-defined and/or unequivocal inflammatory and/or immune responses

2. Applying outcome measures (readout parameters) with robust effect sizes to assess inflammatory and immune responses that permit group numbers to be minimised

3. Limiting control and experimental groups to the minimum needed to generate unequivocal tests of specific research questions

4. Applying multiple readouts in individual experiments to assess inflammatory and immune responses.

These principles will be applied whenever possible to reduce the number of mice required to conduct unequivocal tests of research questions.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding programmes will be designed to ensure maximum usage of mice generated. Pilot studies will be conducted to estimate effect sizes will be conducted if no published information on a specific approach is available. Advice from bioinformatics/statistics experts will be sought if larger scale studies are undertaken. For initial pilot studies to test novel scientific hypotheses group sizes will be 3-5, or will be based on comparable published studies (if available). Multiple parameters will be measured using the same mice when feasible, providing that this incurs minimal increase to health burdens. Some assays can be applied multiple times to the same mice without increasing health burdens excessively, including non-invasive imaging methods to detect tumour growth (CT scans) and mechanical nociception assessments. All data generated will be made available to the research community in a timely fashion to reduce the need for other groups to repeat the studies. Published outcomes from the program of work will follow the ARRIVE guidelines to facilitate subsequent studies by other research groups using these models.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Humans and mice are mammalian species with long periods of shared evolutionary history. As a consequence, humans and mice have highly evolved immune systems with many shared immune cell types with equivalent functional attributes. Murine models of many clinical diseases have been developed and are widely accepted and widely used in the research community. The ability to manipulate specific biological pathways in selective cells is a key factor making mice a suitable species for the proposed research on disease progression and treatment. Moreover a large array of established reagents and databases applicable to mice are other factors promoting their suitability for research on clinical diseases and developing new therapies.

Mouse models selected are suitable to address the four Research Objectives in this project for the reasons described below. Figure 2 indicates how each model is integrated into the four Research Objectives and the overall Project Work Plan.

Inflammation and immune response models (Research Objective 1, Protocol 3): Reagents that incite or modulate inflammatory and immune responses are used to identify pathways driving disease progression, therapeutic responses, and/or therapy resistance. Many reagents (chemicals, toxins, adjuvants, antigens, etc.), environmental factors (infections, allergens, food, exercise, microbiome) and manipulations (wounds, transplants, cells)

cause tissue inflammation. Inflammation activates immune cells but in vivo outcomes (immunity or tolerance) depend on complex physiologic interactions in tissues that cannot be fully reproduced ex vivo. Inflammation stimulates immune responses but often incites immune regulatory (suppressive) processes in parallel and/or in sequence. Regulatory responses may protect tissues from immune-mediated injury but may allow pathogen-infected or malignant tissues to evade protective immunity, leading to persistent infections or cancer, respectively. On the other hand, defective immune regulation may lead to immune-mediated injury of healthy tissues and donor-specific immune responses destroy healthy transplanted organs and tissues, unless immune responses are suppressed by therapy. Inflammation also enhances local pain sensitivity and sustained inflammation causes neurologic and metabolic dysfunction, leading to fatigue and behavioural disorders. Relative to immunogenic responses, immune regulatory (tolerogenic) responses remain poorly defined and are difficult to model using non-animal based approaches due to the complexity of tolerogenic processes and the need to measure immune inhibitory responses. Hence, a major research objective in this project is to identify regulatory pathways that suppress inflammatory and immune responses, which may contribute to disease prevention, progression, resolution, or therapy resistance.

Chronic Infection Model (Research Objective 2, Protocol 4): A natural mouse leukaemia virus (MuLV) chronic infection model was selected because MuLV infections replicate multiple clinical features of retroviral infections in humans (e.g HIV-1 infections that cause AIDS). In recent preliminary work, we found that mice with pre-existing chronic infections (MuLV) are highly resistant to effective treatments that eliminate dermal tumours. As many patients with cancer have pre-existing inflammatory conditions, this finding prompts the need for further research to evaluate how MuLV infection causes resistance to effective cancer treatments.

Cancer Models (Research Objective 3, Protocols 5 & 6): Cancer models were selected because they replicate multiple clinical features of cancer in patients. In recent published studies, (Lemos et al., 2016 Cancer Research and Lemos et al., 2020 J Immunotherapy of Cancer) we described refinements to standard mouse dermal models that better replicate key clinical barriers to effective cancer immunotherapy observed in patients, including late cancer diagnosis, robust immune checkpoints and adaptive therapy resistance. These experimental refinements improve the clinical relevance of mouse dermal models for research on how immune responses that protect developing tumours and how to reduce therapy resistance. In the course of this project, laboratory staff worked closely with local technical and veterinary staff and the Home Office Inspector to improve detection and management of unanticipated adverse effects. For example, we found that novel treatments with STING agonists eliminated tumours but caused transient ulceration at sites of tumour growth due to robust inflammatory and immune responses required to eliminate tumours. In cooperation with technical, veterinary and Home Office staff we developed a novel scoring system to detect and manage adverse effects experienced by tumour-bearing mice subjected to effective (curative) treatments. Aging APCmin mice

develop spontaneous colon carcinomas that replicate multiple features of clinical colon cancer (Protocol 6).

Autoimmune Disease (AD) Model (Research Objective 4, Protocol 7): AD models were selected because they replicate multiple clinical features of equivalent clinical syndromes that manifest in AD patients. The collagen-induced arthritis (CIA) model is the preferred model to study rheumatoid arthritis (RA) and test/validate novel therapies for RA, as the CIA model replicates clinical RA more accurately than other antigen-induced arthritis (AIA) models. However, AIA models may be refined to induce monoarthritis (not polyarthritis seen in clinical disease and the CIA model). and are also useful for discerning disease mechanisms because B6 mice (and hence many GA strains) may be used in AIA models, while the CIA model is confined to susceptible strains such as DBA/1 mice.

Why can't you use animals that are less sentient?

Less sentient, non-mammalian species and immature mice do not have highly evolved or mature immune systems with immune cells and functional attributes that replicate how the human immune system works. Terminal anaesthesia is not appropriate to study inflammatory and immune responses driving chronic inflammatory diseases or therapeutic responses because these processes develop over long periods (weeks to months).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all models, general refinements to minimise suffering (harms) are listed below:

1. Severity will be limited using experimental endpoints based on defined scoring criteria including general health status and disease specific parameters. Goals are to restrict suffering to the minimum necessary to (a) replicate clinical symptoms experienced by patients and, (b) generate high quality outputs that allow rigorous testing of scientific hypotheses under investigation.

2. Treatments and study durations are restricted to the minimum needed to address stated research objectives and to ensure scientific rigour.

3. Low stress handling techniques are used, including tunnel handling during cage change and observations whenever possible.

4. Mice are monitored regularly to identify distressed animals. Supportive care is provided to mice that lose significant body weight or exhibit other adverse effects indicative of distress and suffering. Supportive care includes providing access to moist food and regular observation. The NVS and/or NACWO will be notified if mice approach the maximum permitted weight loss specified in each Protocol (range 20%-30%). Mice will be killed humanely if weight loss does not reverse in reasonable timeframes, based on model-specific criteria and other general health status scores, as specified weight loss limits are approached. On rare occasions, it may be necessary for some control groups to reach the

upper weight loss limit to document effective therapeutic responses in vehicle control groups.

5. Humane endpoints, adverse effects and control measures are described for each Protocol. Mice are killed by a Schedule 1 or non-Schedule 1 method if humane endpoints are reached. Specific justification for the need to allow some mice to reach humane endpoints are as follows. First, on rare occasions it may be necessary for control mice with disease that do not receive therapy to reach humane endpoints in order to document effective therapeutic responses in experimental mice with disease that are given therapy. Second, it may be necessary for some mice that receive novel experimental therapies to reach humane endpoints to validate therapeutic responses rigorously. This is more likely when therapies are applied at later stages in disease progression to model clinical situations accurately. For example, numerous therapies block tumour growth and promote remission and full recovery if therapy is initiated before overt dermal tumours form following engraftment of tumour cells. In many cases, the same therapies fail to control tumour growth if applied after overt tumours form, or when tumours are more developed. Hence, delaying treatments may be necessary to model challenges faced when treating patients due to late diagnosis of cancer when tumours are well developed and more resistant to therapy. Laboratory staff are highly experienced in detecting and managing unanticipated adverse effects arising from complex disease induction and therapy procedures, as illustrated by our effective cooperation with local technical and veterinary staff to develop a novel scoring system to monitor mice with dermal tumours given curative treatments that induced transient ulceration at sites of tumour growth.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance on best practices will be sought from the scientific literature and agencies promoting the 3Rs such as NC3R and ARRIVE.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Updating and implementing advances in the 3Rs will be conducted by reading relevant scientific literature and monitoring sites that provide information about advances in the 3Rs, including the UKRI National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs; www.nc3rs.org.uk) and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (www.arriveguidelines.org)

126. Colorectal cancer initiation and progression

Project duration

5 years 0 months

Project purpose

Basic research

Translational or applied research with one of the following aims:

- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Colorectal cancer, Stem cells, RNA metabolism, Cancer driver genes, Cancer metastasis

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Colorectal cancer is the second commonest cause of cancer related mortality and there is a pressing unmet need for the delivery of new therapies for its treatment. Determining how tumours initiate and progress is critical for understanding tumourigenesis and guiding therapeutic development. The aim of this research programme is to identify potential therapeutic targets by determining the mechanisms important for tumour initiation and progression.

During this research programme we will use appropriate, well-defined mouse models to address 2 aims:

1) The role of changes in how RNA is processed in cancer

2) The identification of novel cancer driver genes

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Expected benefits for objective 1.

My research has the potential to define novel cancer mechanisms. This will increase our understanding of how tumours form and spread and open new therapeutically exploitable opportunities.

Expected Benefits for objective 2.

The identification of novel mutations that drive cancer will provide great benefit to the understanding of how tumours form and spread. It has the potential to identify mechanisms important for tumourigenesis. This in turn may lead to the development of novel therapeutic strategies for targeting colorectal cancer.

What outputs do you think you will see at the end of this project?

The main outputs of this project will be in peer reviewed publications describing the results of our work. These will describe cancer promoting mechanisms and the genetic drivers of colorectal cancer. In addition, the results of this work may lead to additional work developing therapeutics that target the mechanisms we identify. We will also communicate our work more broadly to a wider audience via science communication events, traditional media and social media outputs.

Who or what will benefit from these outputs, and how?

This work will broaden our understanding of cancer driving mutations and mechanisms. In the short term this will benefit the scientific community, who will be able to incorporate and build on our research in their own research programmes. In the longer term, our work has the potential to inform and direct new therapeutic development for the treatment of colorectal cancer. This may lead to patient benefit and wider economic benefits.

How will you look to maximise the outputs of this work?

All work carried out during this project will be published in open access, peer reviewed journals. All data produced during the project will be made freely available to other research groups to help inform their own research. We will also collaborate with other groups to ensure our work is carried out in the most effective way. We will discuss our work during scientific conferences and in public engagement events open to the wider public. Upon publication, we will communicate our findings more broadly using social media and traditional media.

Species and numbers of animals expected to be used

Mice: 15,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

As colorectal tumours are very complex and have a variety of different components e.g. tumour cells, immune cells, supporting cells (stroma) and blood supply, it is not possible to mimic these processes outside of the body. Experiments in animals better recapitulate these effects on how tumours grow and as such it is important to study it in this context. The involvement of the tumour support system and the immune system on cancer cell development and response to therapy is well documented and highlights the need to use mouse models for these studies.

We will use primarily adult mice for our experiments as the genetic models we will utilise can be induced at the adult stage.

Typically, what will be done to an animal used in your project?

In a typical experiment mice will be bred to carry an inducible genetic allele that causes cancer development and additional alleles that we wish to test for a role in cancer development. When the mice reach 6-8 weeks old the genetic mutations are 'induced' by injecting with an agent such as tamoxifen. Mice are then aged until symptoms of tumourigenesis arise and then humanely culled. Samples are taken and analysed to determine the function of the gene of interest in tumourigenesis.

What are the expected impacts and/or adverse effects for the animals during your project?

The primary adverse effects on the mice in this research programme are the development of intestinal/colonic tumours.

When intestinal tumour burden is high in mice, mice become anaemic (as shown through paling of the feet) and begin to lose weight. In around 10-20% of mice rectal bleeding is expected. This occurs due to the presence of small colonic/rectal tumours and is well tolerated by the animals (they show no other obvious signs of distress). Close monitoring will occur after the observance of anaemia and/or bleeding and mice will be humanely culled when they show signs of moderate discomfort. This is described as three of the following symptoms - weight loss over 20%, subdued behaviour, stop interacting with their cage mates, anaemia, sustained rectal bleeding with visible blood on fur and sustained diarrhoea for over 72h. Other mice will be culled at pre-defined time points according to the experimental protocol at which time tissues will be taken for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild severity (75%)
Moderate severity (25%)

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Colorectal tumours are very complex and have a variety of different components e.g. epithelial tumour cells, immune cells, supporting cells (stroma) and blood supply it is not possible to mimic these processes outside of the body. Only in vivo approaches recapitulate micro environmental effects on tumourigenesis and as such it is necessary to study it in this context. The involvement of the tumour microenvironment and the immune system on cancer cell development and response to therapy is well documented and highlights the need to use mouse models for these studies.

Which non-animal alternatives did you consider for use in this project?

We, and others, have developed a number of ex vivo tumour and tissue organoid culture techniques that will be used in parallel to these studies. These alternative models complement the use of animals and can be used for initial target identification and mechanistic analyses. In particular we have developed models for CRISPR/Cas9 screening in these ex vivo models in order to refine targets for in vivo testing. For example, we routinely use CRISPR/Cas9 to screen for candidate genes that impact on tumour organoid growth but do not impair normal intestinal organoid growth. This approach allows us to determine candidate genes that not only are likely to suppress tumour growth in vivo but not detrimentally impact on normal mouse physiology. We also routinely carry out bioinformatics approaches, for example mining human gene expression datasets to help determine human disease relevance to selected target genes. These approaches reduce the number of animals used by focusing their use on addressing the key questions throughout the proposal. We have utilised this approach under our current PPL to successfully stratify candidate genes for analysis. This has led to the identification of a number of cancer driver genes with reduced animal usage than would have occured without these ex vivo approaches.

Why were they not suitable?

Despite acting as disease relevant models and being useful for initial target identification experiments organoid models fall short of fully recapitulating the complex environment found within tumours. In particular, they lack immune, stromal and vasculature involvement and cannot be used to model more complex tumour phenotypes such as tumour initiation and metastasis. For these reasons these models fall short of replacing the need to carry out these experiments in animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Power analyses, based on our past experience, have been performed to estimate the number of mice required to generate significant results.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals used in this project we took a number of steps:

1) Power analyses were performed to ensure that we use the minimum number of mice to generate significant results. These were based on our past experience.

2) Embryos/sperm are frozen from lines not immediately required for experimental studies.

3) Tumour metastasis is a highly complex process that occurs following the acquisition of multiple genetic mutations. The only reliable autochthonous genetically-engineered mouse model that gives rise to liver metastasis carries 4 separate genetic alleles (VilCreERT2 P53fl/fl KrasG12D Notch-ICD). Therefore, the complex breeding to generate such models and add additional alleles to test effects carries a large animal usage burden (over 1000 mice would be required to generate cohorts for analysis). Organoids from these mice can be genetically altered using CRISPR/Cas9 and successfully re transplant at high efficiency (~80%). Therefore, the use of orthotopic transplantation models in syngeneic mice is a viable, disease relevant alternative with a small fraction of the animal usage.

4) By developing a model for in vivo genome editing using CRISPR/Cas9 in adult mice we can significantly reduce animal numbers compared to the use of traditional breeding programmes to generate mouse lines carrying multiple genetic mutations. Similar to the orthotopic model outlined above a large animal breeding burden is needed to generate mouse cohorts for testing the function of genes in tumour initiation. Our standard tumour

initiation model (VilCreERT2 Apcfl/+) requires over 100 mice to cross another allele to homozygosity and generate cohorts for analysis. By generating colonies of VilCreERT2 Apcfl/+ Cas9 mice we can reduce this as all mice generated can be used in experiments.

Throughout our experimental design we have utilised information derived from experiments carried out under our current PPL to inform this process.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1) Breeding plans are optimised for efficiency

2) Any tissues generated from previous experiments are archived and stored appropriately therefore ensuring that repetition of experiments is not necessary.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically engineered mouse models will be used throughout this project. In addition, orthotopic transplantation of organoids into syngeneic recipients and direct in vivo CRISPR/Cas9 editing of the colon will be carried out. Procedures will include injection or feeding with inducing agents (eg tamoxifen, doxycyclin), injection with labelling agents (eg BrdU), induction of colitis (DSS) and treatment with candidate therapeutic agents.

Why can’t you use animals that are less sentient?

These models have been chosen because the mutations that they carry are those associated with the human disease allowing us to recapitulate the same genetic mutations observed in human cancer. No other models are currently available to perform this analysis and lower species such as fish or flies do not accurately recapitulate the human disease. Indeed, a number of studies in fish have demonstrated fundamental differences in the mechanisms of Wnt signalling activation following loss of Apc so this model is inappropriate for modelling human colorectal cancer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible cre-loxP systems will be used to specifically target the tissue of interest thereby reducing off-target adverse effects in the animal. The group will ensure that all animals receive the highest standard of care and appropriate social, environmental and behavioural enrichment such as bedding, tubing and group housing and will be provided. Close monitoring of tumour development will ensure animal suffering is kept to a minimum. We have vast experience of these cancer models and the clinical signs that develop and can therefore ensure that suffering is kept to a minimum. For the genetically engineered mouse colorectal tumour models animals will be euthanized when they reach the clinical endpoints outlined in Appendix A. Reaching these clinical endpoints is extremely important to allow disease progression to a stage where it reasonably mimics the human disease and to ensure all animals with different genetic alterations are taken at an equivalent disease stage thus allowing accurate comparisons between them. Additionally work carried out to date under our current PPL has shown that sacrifice of the animals at this stage allows us to obtain statistically significant results when studying the role of key proteins in colorectal tumour development.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

For all our studies we will refer to the Guidelines for the Welfare and use of animals in Cancer Research (Workman et al, 2010) and ensure best working practice. We will use all the refinements (1-

10) recommended in this document. We will also refer to PREPARE and ARRIVE guidelines: https://journals.sagepub.com/doi/10.1177/0023677217724823
https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1000412

and the following resources: https://www.nc3rs.org.uk/experimental-design
https://acmedsci.ac.uk/policy/uk-policy/animals-in-research

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Via the 3Rs website: https://www.nc3rs.org.uk and communication from animal facility management.

Staff in my lab will be kept informed of any advances in 3Rs and will implement them. Changes to protocols as a result of these advances will be implemented via license amendments.

127. Tuberculosis immunity and vaccine development

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

vaccines, infectious diseases, immunology, diabetes

Animal types	Life stages
Mice	juvenile, adult, pregnant, aged, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project are to:

a) understand the complex interaction between Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), and the host immune system, and how diabetes and co-infections e.g. cytomegalovirus (CMV), affect these interactions

b) develop and evaluate vaccines against TB and other pathogens, for which there are currently ineffective vaccines or no vaccines at all.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Tuberculosis (TB) is the leading cause of death from a single infectious agent, killing more than a million people a year. There is an urgent need for better control measures and the most cost effective way to control any infectious epidemic is with effective vaccination.

There is only one available vaccine against TB, but it is able to prevent only 5% of all vaccine preventable deaths. For this reason a more effective vaccine is urgently required that will also target an increasing diabetic population and people with co-infections.

What outputs do you think you will see at the end of this project?

Detailed understanding of immune responses to tuberculosis and identify responses that correlate with protection

Understand how protective immune responses are altered by diabetes and co-infections

Develop more efficient vaccines against tuberculosis and related diseases, and understand how these vaccines interact with the immune system.

Who or what will benefit from these outputs, and how?

It is estimated that one-quarter of the world's population is currently infected with Mycobacterium tuberculosis. The majority of infections are in the developing world, where co-infections with HIV, cytomegalovirus (CMV) and the prevalence of diabetes result in worse disease outcomes. The long- term aim of this work is to develop a vaccine that is effective in all populations and in all risk-groups, and will help control the TB epidemic.

Our short-term aim, is to identify immune responses that correlate with protection, and evaluate a number of vaccines for their immunogenicity and protection. These findings will help the scientific community, and in particular the TB vaccine field, by ensuring that vaccines in development are able to induce these protective immune responses. In addition, information about how immune responses are altered when diabetes is present and when there is an underlying co-infection, will inform all vaccines in development. This was emphasised in the current COVID-19 pandemic, where people with hyperglycaemia/diabetes, had worst disease outcomes. For this reason, vaccines should be equally effective in these populations.

How will you look to maximise the outputs of this work?

We will publish our findings, both positive and negative, in peer-reviewed journals and we will present in meetings and consortia.

We have a number of on-going clinical trials, and a large number of stored samples and there will be a constant feedback between the two sides to maximise the impact and confirm the relevance of our findings. In addition, we have a number of collaborations (human and veterinary) that we will be sharing our results and techniques with.

Species and numbers of animals expected to be used

- Mice: 16,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for this work as they are similar to humans in the main features of the innate and adaptive responses to TB. Just like in humans, TB primarily infects the lungs. In addition, there is an abundance of immunological reagents and assays that allow extensive characterisation of any aspect of TB pathogenesis, much more than in any other animal species. In some instances, we will be using genetically altered mice, that lack parts of the immune system, in order to help understand what is their role in vaccination and in protection.

We will use juvenile and adult mice as they have a fully developed immune system.

Typically, what will be done to an animal used in your project?

Immunogenicity experiments: Mice will be typically immunised on up 3 occasions and immune responses will be measured at pre-defined end points. Hyperglycaemic mice will also be immunised with promising vaccines to understand if and how immune responses differ. Similarly, mice will be

inoculated with MCMV before immunisation to explore the impact on vaccine immunogenicity and efficacy.

Efficacy experiments: To investigate the protective capacity of vaccines, animals will be first immunised with our vaccines and then challenged with Mycobacterium tuberculosis. We will be comparing the bacterial load of the vaccinated compared to unvaccinated control animals. On rare occasions we will challenge vaccinated hyperglycaemic mice, to ensure that promising vaccines are still protective.

On rare occasions, we will be using genetically-altered mice, that lack parts of the immune system, to understand what is their importance in infection and vaccination.

What are the expected impacts and/or adverse effects for the animals during your project?

Our immunisation experiments are expected to have only mild effects that are transient, similarly to human vaccination. In the majority of cases, animals will be sedated to avoid stress and pain associated with the procedure.

For challenge experiments, animals will be monitored for signs that are associated with disease progression, such as weight loss, loss of activity. Typically challenge experiments are completed before any signs of systemic disease, but animals will be closely monitored throughout these experiments to ensure that humane endpoints are not exceeded

Hyperglycaemic mice will have a higher blood glucose level compared to animals with normal levels. Adverse effects include increased urination, higher water intake and weight loss. These animals will be monitored for both blood glucose levels and weight.

Infection with MCMV results in latent infection that is held in check by the immune system. MCMV might cause short-term transient, non-specific illness and therefore animals will be closely monitored after infection, and in the days following infection, to ensure complete recovery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 10% subthreshold
- 60% mild
- 30 % moderate

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although human challenge models do exist for other diseases such as malaria, safety and ethical concerns over human TB challenges have limited their development. Moreover there are currently no immune responses that correlate with protection, and for this reason the efficacy of vaccines needs to be assessed in animals. Mice are similar to humans in the main features of the innate and adaptive responses to TB. Just like in humans, TB primarily infects the lungs. There is currently no in vitro system that can mimic the complexity of the immune response.

Which non-animal alternatives did you consider for use in this project?

Human challenge studies:

a) We have performed a human challenge study, using BCG vaccine, as a surrogate for M.tuberculosis challenge,

b) on-going challenge study using aerosolised BCG as surrogate for M.tuberculosis challenge.

In vitro assays: human cell samples and blood post-infection and post-vaccination, in vitro infections such as mycobacterial growth inhibition assays (MGIA) to test the efficacy of vaccines, in vitro infections to identify promising antigens vaccine preparations

In silico modelling e.g. bioinformatics, to identify promising antigens to include in vaccines.

Less sentient animals such as Drosophila melanogaster, amoeba Dictyostelium discoideum and zebrafish

Why were they not suitable?

Human challenge studies: a) although this study was able to show the protective ability of some vaccines, the route of infection was different to the natural aerosol route of M.tuberculosis. For this reason, the following study (b) is investigating the aerosol route of infection. Although these studies are very informative, they will not be able to replace animal challenge experiments that aim to compare different vaccine candidates

In vitro and in silico assays: we use human cell samples post-vaccination and pre and post- infection to identify promising antigens to include in our vaccines. This reduces the number of vaccines to be evaluated in animals but there is still the need for vaccination and evaluation of vaccine efficacy in animals

Less sentient animals: the lack of an adaptive response, a key part of the immune system induced by vaccination, is absent in these animals. In addition, although the zebrafish is more similar to humans in their immune system, they do not have lungs, the primary organ that is infected by M.tuberculosis

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To generate some transgenic mice for our experiments, we need to perform complex genetic crosses; in addition we will need to backcross genetically modified animals to parental strains to minimise genetic drift.

For the animals to be used in our experimental protocols, we estimated our number on past experience and on the number of projects that we have on-going. We have performed

power calculations based on past data to identify the minimum number of animals to observe differences in our experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use in vitro assays to screen promising antigens to include in our vaccines. For example, we screen antigens on human blood from TB infected individuals, and perform in vitro infection experiments, followed by mass-spectrometry. This step minimises the number of vaccines that need to be tested in mice.

Rigorous statistical calculation to identify the appropriate number size to ensure that the minimum number of animals are used but at the same time ensure that each study is sufficiently powered to provide a meaningful result.

Minimise variation by controlling for cofounding factors such as sex, age, environment. Lower variation means that less animals are required per group.

Trying to address multiple questions within one experiment. For example, assess immunogenicity and protective efficacy of a vaccine by performing an in vitro challenge when possible

We will be conducting our experiments to comply with the ARRIVE guidelines when publishing

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Cryopreservation of mouse strains when not in use

Pilot studies with small number of animals, when a new vaccine regime or animal strain is used for example, to understand the variability within each group

We will use in silico modelling to select antigens to be included in our vaccines

We will be sharing samples with collaborating groups who investigate different aspects of the same immune responses e.g. metabolomics, epigenetics

We will use good colony management to ensure that only those cohorts required for experiments are produced. Breeding data is available from our mouse database to enable effective breeding calculations to be conducted.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse strains that are commonly used for TB research will be also be used for this project. We will use non-genetically modified strains such as C57BL/6, BALB/c, CB6F1, all of which are quite resistant to TB infections. Challenge experiments with these strains allow us to detect the effect of vaccination before any systemic signs of disease develop. In some instances, genetically modified animals will be used to explore the role of particular aspects of the immune system in vaccination and TB protection.

The methods that we will be using are: immunisations and challenge experiments.

To investigate the effect of hyperglycaemia on vaccine immunogenicity and efficacy, we selected two mouse models that best represent human type 2 diabetes. One is an altered diet model where mice are fed on high fat/sugar diet and one a transgenic mouse model. We will not be inducing diabetes in these mice, but moderate levels of hyperglycaemia to mimic subclinical diabetes. Methods will include the induction of hyperglycaemia, immunisations and in some instances challenge experiments.

Why can't you use animals that are less sentient?

The mouse model is the least sentient model with a similar immune system to humans. Just like in humans, TB primarily infects the lungs. In addition, there is an abundance of immunological reagents and assays that allow extensive characterisation of any aspect of TB pathogenesis, much more than in any other animal species.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Particularly when testing the susceptibility to TB infection of new animals strains, co-infections or in hyperglycaemic mice, we will perform pilot challenge experiments with the non-pathogenic BCG mycobacterial strain.

Our state of the art aerosol challenge equipment, allows us to perform infection experiments with really low M.tuberculosis doses, which a) are more relevant to the human situation and b) cause infection with a lower bacterial burden

In vitro challenge assay, is currently optimised and used in our laboratory. This assay, referred to as mycobacterial growth inhibition assay (MGIA), has the potential to replace in vivo challenge experiments. We will use this assay as a first step for screening new vaccines or investigating the impact of mycobacterial infection on new genotypes.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the ARRIVE guidelines and the NC3Rs Experimental design assistant when setting up new experiments. The Design of animal experiments, 2nd edition, by Michael Festing et al will be used to help us design our experiments and online statistical tools such as G*power will be used to calculate group sizes. In addition, The Handbook of Laboratory Animal Management and Welfare, Sarah Wolfensohn and Maggie Lloyd, Blackwell publishing, for guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have members of our team that are part of the local ethics committee and members that are funded by the NC3Rs to develop assays that are a significant refinement to the standard protocols. We regularly attend internal 3R's meetings and use the NC3Rs website to keep up to date with the more recent advances in the 3R's

128. Impact of hormones and inflammation on reproductive health and disease

Project duration

5 years 0 months

Project purpose

Basic research
Key words

hormone, reproduction, inflammation

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to understand how signalling molecules called hormones control the function of immune cells in order to understand how this affects women's reproductive health and to find out if this could be targeted in new therapies for women's health disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

One in three women in the UK will suffer from a reproductive health problem such as endometriosis or heavy menstrual bleeding. These common disorders cause life-changing symptoms. Reproductive disorders impact on the health and wellbeing of individuals and carry a high cost to both women and society.

Despite its clear importance, lack of funding has hampered research into women's reproductive health, and this has prevented development of new treatments. Current treatments for conditions such as endometriosis or heavy menstrual bleeding include fertility-ending surgery or medical therapies that have unwanted side effects.

The womb must repair as part of each monthly cycle (menstruation) in order to maintain healthy function. This is essential for ongoing fertility and reproductive health. Repair is abnormal in reproductive health disorders and this may be caused by unbalanced hormones and increased inflammation caused by immune cells. Hormones are a type of messenger signal that is released into the blood and travels to different organs to change their function. Specific types of hormone are required for reproductive function; these are known as sex steroids. In females, the womb changes its function in response to sex steroid hormones that stimulate growth (estrogens) and remodelling (progestogens) as part of normal reproductive function. Androgens are a type of sex steroid hormone that are usually associated with male reproductive function but we have found that androgens are also important for normal function of the womb. Androgens can alter wound repair in other tissues such as the skin and some studies suggest this is because they can change the function of immune cells. We do not fully understand how hormones can affect immune cells and this has not been investigated in the context of womb repair or women's reproductive health. We know that unbalanced sex steroid hormones are associated with abnormal immune cell function and reproductive health disorders.

We want to understand how hormones control immune cells in order to understand how this could contribute to women's reproductive health. If we can understand this we believe it could help us to develop new therapies for women's reproductive health disorders.

What outputs do you think you will see at the end of this project?

This project will assess how hormones and immune cells control womb function in health and disease. This work will develop new understanding that will be shared with other researchers by presenting our findings at meetings and by publishing our results in journals and online via social media/websites.

This research will help us to better understand how the womb works, which will help us to develop new ways to improve women's reproductive health. New treatments will be developed by testing targets found from this project in human cells/tissues.

Who or what will benefit from these outputs, and how?

This project will benefit clinical doctors and scientists with an interest in women's health who will gain new knowledge relevant to their research.

The results will also be of interest to the pharmaceutical industry, clinicians and scientists who are keen to develop new treatments for women's health problems. One key opportunity involves using drugs already developed for treatment of inflammatory and hormonal disorders but as a treatment for women's reproductive health disorders. This

approach may be of particular interest to patients as these drugs could be developed into new treatments more quickly than entirely new drugs. These long-term benefits may come from the research conducted as part of this project.

How will you look to maximise the outputs of this work?

Research outputs will be shared broadly with other researchers by presenting research findings at meetings and publication of research findings in academic journals and online via social media and websites. This approach will help to ensure the benefits of the research can reach key stakeholders.

The research outputs in this project may benefit other researchers both locally and internationally and help to shape research assessing the role of hormones and immune cells in other contexts. Relevant information will be shared with collaborators and colleagues through departmental research meetings where formative/unsuccessful aspects of research can be shared and improved, local/national research meetings and via publication in journals. Research, including data from unsuccessful approaches will be made available at the earliest opportunity via preprint servers such as Biorxiv (https://www.biorxiv.org/).

Species and numbers of animals expected to be used

- Mice: 7950

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Normal functioning of the womb (endometrium) is essential for reproductive health. The endometrium is a complex organ and a full understanding of the how different cell types interact and how signals called hormones control these cells requires the use of animal models. Importantly, our studies, and those of others, have demonstrated that the endometrium in adult female mice is strikingly similar to human tissue and they share the same cellular compartments. Important for this proposal is the ability to model features of human menstruation (period) which involves endometrial tissue breakdown, inflammation and repair. We have developed a mouse model that can mimic this in a consistent manner and the mouse model of endometrial repair shares similar key features to human.

The process of endometrial repair is linked to fertility and pregnancy and therefore can only be modelled in adult mice. Moreover, the immune system and particularly the part of the immune system we wish to focus on (macrophages) is well defined in mice and shares similarities with human in health and disease. Powerful research tools are available if you use mouse models including the ability to change expression of key factors or to label

specific cell types using genetic approaches. Studies in mice therefore provide the ideal platform for our investigations for which we have well validated models of endometrial repair that are appropriate for the aims of this project.

Typically, what will be done to an animal used in your project?

The experimental work will involve some minor surgical procedures and delivery of hormones by injection or slow releasing capsules placed under the skin. Procedures are classified as either moderate or mild in severity. These procedures will allow modelling of endometrial breakdown and repair as well as modulation of hormonal and inflammatory processes. Pain will be controlled during surgery by general anaesthesia and post surgically by painkillers (analgesics). Animals will be monitored during the post-surgical period and further analgesia will be administered if animals exhibit signs of discomfort. Surgical procedures have short duration, typically less than 10 minutes. Typically, an experiment will involve up to three short surgeries within a three-week protocol.

There are two main types of surgery; removal of ovaries (ovariectomy) and placing capsules under the skin that release hormones, which are performed under general anaesthesia. Ovariectomy involves making a small incision in the skin, making a small hole on the body wall, holding the uterus with forceps and removing the ovaries. The opening in the skin is then closed using surgical clips. This procedure takes 7 minutes on average and mice have actively recovered (awake and moving freely) within around 10 minutes. Insertion or removal of capsules involves making a small incision in the skin and placing or removing the capsule under the skin. This takes less than 5 minutes and recovery within 10 minutes. In each case, animals recover quickly and return to normal behaviour after procedure and anaesthesia. These procedures allow hormone signalling to be changed in order to mimic changes that take place in the menstrual cycle in women that allows us to better understand endometrial function and repair.

Endometriosis is the presence of endometrial cells/tissue at sites other than the uterus, usually on organs or tissue inside the pelvic abdominal cavity. In women, these deposits form lesions and this is associated with symptoms of infertility or pelvic pain. We have developed a mouse model that mimics some features of endometriosis in order to help us to understand the factors that allow lesions to become established and to grow in the pelvic cavity. Our model generates endometrial deposits, that establish in the pelvic cavity and have a similar architecture to endometriosis lesions found in women. This procedure involves transferring endometrial cells/tissue by injection into the pelvic cavity of the mouse and allowing these deposits to attach to the peritoneal wall over a period of 2-3 weeks. This procedure is associated with changes in immune cells, particularly macrophages, which are similar to changes that are seen in women. The procedure is well tolerated with no obvious adverse effects.

We will monitor behaviour of mice on this protocol to ensure that model or any interventions do not have a negative impact on behaviour. Our previous studies have used

state-of-the-art movement monitoring techniques and found that mice with endometriosis lesions behave no differently from control mice.

Thus, although this model is useful for understanding the role of inflammatory cells and hormones in establishment of lesions it does not recreate pain symptoms that are seen in women.

Some previous studies suggest that experimentally-induced endometriosis can cause animals to become more sensitive to unharmful stimuli, which can be considered a 'pain-like' behaviour. Although we have not seen this in our experience, we will measure this by using established tests such as von Frey test. Von Frey test uses fine plastic strands of different widths that are applied to the hind paw and if the rodent withdraws, licks or shakes the paw then a positive response is recorded. If an animals sensitivity is increased this response will happen with finer strands (smaller width) than in control animals.

Taken together, the majority of animals in this project will undergo procedures to allow us to mimic events that occur in the menstrual cycle in women in order to investigate the impact of hormones on macrophage function in the endometrium. Endometriosis will be experimentally induced in some animals in this project in order to understand of how hormones and macrophages can contribute to establishment and growth of endometrial deposits as this can help us to better understand what causes disease in women.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected, and appropriate measures have been taken to eliminate the possibility of such complications. However, infections can occur after surgery in approximately 1% of animals. Risk of infection will be minimised by good surgical and aseptic technique. Pain post-surgery may occur and analgesia will be administered following surgical procedures to minimise this impact. Animals will be monitored during the post-surgical period and further analgesia will be given if animals show signs of discomfort. All substances will be given at doses known to be non-toxic based on our experience and reported dosages. At the end of procedure, animals will be humanely terminated and tissues collected for analysis.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The experimental work will involve some minor surgical procedures and administration of hormones via injection or as slow releasing pellets placed under the skin. The procedures are classified as either mild (15%) or moderate severity (85%). Most animals will be classified as moderate.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Menstruation and endometriosis only occur in women and higher primates (apes and some monkeys). Using our understanding of reproductive biology we have been able to develop mouse models of menstruation (endometrial wounding and repair) and endometriosis. Our models have provided an ideal platform for improving our understanding of how the endometrium functions in health and disease.

Complex interactions between different cell types cannot be fully explored without the use of an animal model. This is particularly true of immune cells and in particular macrophages which are controlled by signals from their tissue environment and therefore do not behave the same way when removed from the tissue and studied in isolation in a dish. Also, because hormones act by signalling through the blood their actions need to be investigated in an animal model.

Which non-animal alternatives did you consider for use in this project?

We have used human tissue specimens recovered from women in the adjacent hospital to study behaviour of different cells in the womb that have informed our experimental approach. This allows us to test cells in isolation, or to investigate samples of tissues taken at a single time point. We have tested single populations of cells and developed co-culture systems to identify relevant cell-type markers and to understand some basic cell interactions.

Why were they not suitable?

Although these studies are informative they offer only limited insight. Isolated cells do not behave in the same way as those within tissues and the complex interactions between different cell types cannot be investigated with this approach. Importantly, the processes we wish to investigate are dynamic and complex and cannot be recreated by in vitro experiments or understood by assessing tissue specimens. Animal models allow us to understand how specific cell types in endometrial tissue function and how they respond to different signals that would not be possible using other approaches.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

An expert statistician has been consulted about the experimental design of the studies and statistical calculations have been used to predict the minimum number of mice to be used. These calculations have been informed by our previous experience using the mouse model of endometrial repair and studies we have conducted investigating the impact of hormones on reproductive function.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experimental design has been informed by NC3Rs experimental design hub resources and will adhere to the PREPARE guidelines on how to design conduct experiments (https://norecopa.no/prepare) which have been developed with the involvement and support of the RSPCA. We referenced guidelines for animal colony management to ensure safe, reliable, and efficient animal breeding of genetically altered mice that we will need to conduct the studies in this project (Jackson Laboratory Resource Manual; Breeding Strategies for Maintaining Colonies of Laboratory Mice).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In vitro work and studies on human tissues/cells will be carried out in parallel with all animal experiments to inform choice of targets for analysis and ensure we investigate mechanisms that have the most relevance to human health. Our studies will have broader relevance to researchers investigating the roles of hormones in regulating inflammatory responses in other tissues and as such sharing of tissue will be encouraged as much as possible. Efficient breeding strategies will be informed by NC3R's Breeding and colony management resources (https://nc3rs.org.uk/breeding-and-colony- management) and by completing specific training in mouse genetics/breeding provided by MRC Harwell (https://www.har.mrc.ac.uk/training/).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The architecture of the reproductive system in female mice mirrors that in women and there are close parallels in hormone regulation of womb function between the two species both of which are mammals. We have developed mouse models of menstruation and endometriosis and validated that they recreate key features of tissue recovered from women giving us confidence that they are robust models for studying these processes and their regulation.

Mouse models allow use of animals in which expression of key factors required for immune cell or hormone function can be changed genetically. This approach is more refined than using chemical mediators or drugs which can have off target effects thereby minimising risk of suffering. This approach will allow us to identify key mechanisms unambiguously ensuring results are robust and meaningful thereby minimising animal use.

The surgical procedures used in this project are well validated and have been used in this context since 2014. We are expert in handling and administering the procedures and therefore animal distress is minimised. In using these methods we carefully monitor animals and ensure appropriate anaesthesia and analgesia is used at all times to minimise pain, suffering and distress. The hormones we use in our methods mimic normal changes that occur in the animals and any additional drug treatments used are at doses known to be non-toxic. At the end of procedures animals will be humanely terminated using Home Office approved methods.

Why can’t you use animals that are less sentient?

The architecture of the reproductive system in female mice mirrors that in women and there are close parallels in steroid regulation of endometrial function between the two species both of which are mammals. Species such as worms and flies do not have an endometrium although they do have factors that are the equivalent of steroid hormones.

We cannot fully explore complex cell-cell interactions in the endometrium using cell culture models. Menstruation and endometriosis only occur spontaneously in women and higher primates, however, we have developed mouse models of menstruation (endometrial wounding and repair) and endometriosis which have provided an ideal platform for understanding how hormones, immune cells and progenitors contribute to these complex processes. These methods require adult mice as mice at this stage have a mature hormone, immune and reproductive system which is necessary to see the appropriate changes and responses that are recapitulated by the model.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

My group will consult closely with our local expertise and utilise the extensive resources provided by the our website to ensure we are following current best practices. As other

more refined approaches become available during the course of this license either through personal communication, publications or veterinary advice, we will investigate their use following discussion with the NACWO, NVS and Home Office Inspector before incorporating them into our plans.

For example, our facility has adopted improved rodent handling methods that reduce animal stress and now provide environment enrichment as standard which we have adopted.

In addition, we have extensive experience of the surgical procedures and required post-operative care and pain management. In order to improve our monitoring we have introduced digital records to ensure all members of the team and facility staff/NVS can access these throughout procedures.

We also plan to evaluate animal behaviour using new non-invasive in-cage systems. Importantly this system is operator blinded and allows repeated (longitudinal) monitoring of a single animal controlling for intra-animal variability and reduction in animal numbers. This will give refined insight into animal experience on our protocols.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will routinely consult the NC3R's website (https://www.nc3rs.org.uk/) experimental design hub resources and will adhere to the PREPARE guidelines on how to design conduct experiments (https://norecopa.no/prepare) to ensure experiments are conducted in the most refined way.

We will also take full advantage of the local Animal Welfare Committee annual seminar day to find out about pioneering developments in best practice and methods to improve animal welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To ensure our experimental approach minimises welfare costs to animals, we will seek to incorporate any new refined approaches, identified via personal communications, publication or veterinary advice, to our studies.

We will also routinely consult the NC3Rs website (https://www.nc3rs.org.uk/) and take full advantage of the Animal Welfare Committee's annual seminar day to find out about pioneering developments in best practice and methods to improve animal welfare.

Any adaptations will take place following discussion with the NACWO, NVS and Home Office Inspector before incorporating them into our plans.

129. Prevention and treatment of sepsis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Infection, Vaccination, vaccine

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to understand how bacteria cause infections, and will use that knowledge to develop vaccines, and other interventions that might prevent transmission or infection progression.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Bacteria can cause mild infections that clear up quickly with antibiotics, but can also cause more serious invasive infections such as bloodstream infections. Although rare, these serious infections can be devastating, with mortalities of around 20% even with modern treatment. A better understanding of how bacteria move from one part of the body to another and from one person to another, combating the immune response, will help us to

develop interventions to prevent these events, and help with vaccine development. In an era where bacteria are increasingly resistant to antibiotics, such knowledge and interventions are much needed.

What outputs do you think you will see at the end of this project?

The project will deliver new information about how infection spreads and help to develop better interventions to prevent transmission and limit the progression of an infection, potentially with new treatments that can act alongside standard antibiotics. The project will increase our understanding about how the immune system responds to infection and is predicted to deliver new vaccines that prevent infection.

Who or what will benefit from these outputs, and how?

Patients and the wider public will benefit the most from such research. Outbreaks of some infections cause huge inconvenience and sometimes deaths. By understanding the scientific factors that influence infection transmission this project will hopefully inform guidance on how to prevent or limit outbreaks. Research might also identify a need to monitor certain strains of infection that are more liable to cause outbreaks.

The proposed work will also assist in development of vaccines, especially noting the increase in bacteria that are no longer responding to antibiotics. For the most common types of bacteria, there are often no vaccines at all. Although vaccines often take at least a decade for development, the recent covid-19 pandemic has demonstrated that it is possible to accelerate vaccine development when necessary.

How will you look to maximise the outputs of this work?

The outputs of this work will be discussed with those who influence policy in public health.

The findings of the work will also be fed back to relevant patient support groups and published in peer reviewed journals. The work will also be presented at national and international meetings, to ensure stakeholders in the wider academic and pharma communities are aware of relevant findings. at an early stage.

Species and numbers of animals expected to be used

- Mice: 4000 although this is a maximum

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Much of our research relies on understanding a normal immune response to infection, and aims to develop vaccines, so it is important to model infection in a mammalian system that has working circulation and immune systems similar to humans. To achieve this, we will use adult mice. Other animals lack the necessary immune cells to make, for example, antibodies and lack the circulation systems of mammals. Furthermore, since many infections affect mucosal membranes and skin, it is important to use a model system that breathes air similar to humans and produces immune responses in surface layers of the body similar to humans. This can only be modelled in mammals and the mouse therefore represents the most conservative and appropriate system to use avoiding use of higher mammals or mammals from the wild. It is possible to use mice that have been modified to better represent humans, or that are deficient in a specific immune factor that is under investigation. Adult mice are required since immune responses and the bacterial living in the gut are fully developed and stable by then, meaning that the outcomes of infection experiments are more reproducible between individuals.

Typically, what will be done to an animal used in your project?

Our project is focussed on infection, therefore all of the protocols include an infection step-other than the preliminary stages of testing a new vaccine or treatment. In all cases we may need to test an animal's baseline level of immunity, and also check what bugs the animal is already carrying; we can do this by taking a small blood or saliva test, and by sampling the nose or mucous membranes beforehand.

In some cases, mice will be infected via the nose, and monitored daily to find out how long the infection is carried in the nose. These infections are usually limited to 7 days as most mice clear infection within a week. Mice are checked daily to see if they are carrying the bacteria. Mice do not get sick or show any symptoms from this infection because the volume administered is tiny.

Some mice may receive an inoculation onto, into or just under the skin, while others might be receive an injection into the muscle in a small volume similar to a vaccination. These mice will usually only be infected for a maximum of 24 hours.

During the infection period, small blood samples may be taken to check for bacteria in the blood. We will weigh animals regularly as this provides an early indication about the infection progression.

Occasionally mice may be anaesthetised during infection to allow us to monitor infection more closely. At the end of any infection model, the bacteria are counted from the site of the original infection and also from as many organs as possible, including the blood.

To understand the effect of novel treatments, or of specific components of the immune response, we will sometimes administer a medicine or antibody to the mice, either before, or during the infection. This might include antibiotics, because it is important to understand how novel treatments work together with routine treatments that patients receive. When we use antibiotics, we choose doses and dosing gaps that are as close as possible to

what is used in humans (the doses are based on weight, therefore we follow doses used for newborns). Most substances are administered intraperitoneally (into the abdomen) or intravenously (into a vein) for easiest rapid spread into the bloodstream. Mice that are infected, are then monitored for progression of infection.

To evaluate vaccines we will need to administer vaccines before any infection. Vaccines are generally made from one or more components of a pathogen, combined with a chemical called an adjuvant, which is a substance that promotes an immune reaction. Vaccines are usually given as injections into muscle or under the skin as in humans and are given on a schedule every 2-3 weeks (usually no more than 3) until immunity develops- this is tested by a blood or saliva test. After the vaccination schedule is completed, mice will be infected and the progress of the infection is monitored, comparing mice that received a test vaccine with those that received a sham (fake) vaccine.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice that experience nasal infection are not expected to undergo any adverse events, although some may experience reduced appetite/eating less for 24-48h; invariably all mice quickly recover from this.

Mice that experience intramuscular infection may experience some pain; although experiments do not normally last more than 24h pain killers will be used if required.

Any mouse that is undergoing a bacterial infection administered into the skin or muscle may show signs of infection, such as eating less, or ruffled fur but these experiments do not last more than 24h.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

Overall for this project, it is estimated that for mice, 40% will undergo mild severity and 60% moderate severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project aims to understand how infection progresses in individuals and between individuals; this can only be modeled in living animals or humans where the circulation is moving, and where breathing is ongoing. Although humans can be used to model infections in so called 'human challenge' models some infections are considered too dangerous for human- while mice appear to tolerate these much better than humans. The project also aims to develop vaccines: Most vaccines being developed aim to protect children early in life. One other problem with human challenge models is that adult humans often have immune responses to the infections being studied, making them poor experimental subjects. Furthermore, having a model system that can reproduce immune responses similar to humans is invaluable. This would not be possible with fish or insects.

Which non-animal alternatives did you consider for use in this project?

To study the immune response to infections, we already use samples from patients with the same infection. We also use human blood cells as a model system although it is necessarily a static system and limited to the cells in a tube- this cannot mimic the dynamic movement of immune cells to an infection site.

Why were they not suitable?

These samples provide very useful information but do not allow us to undertake experiments or test new interventions or vaccines.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We use statistics to help us decide the lowest possible number of mice needed to get a meaningful answer to a specific question. The projected number of animals reflects our best estimate of the number necessary to achieve the proposed scientific objectives. We also considered the number of mice funded in our new grants, as well as an estimate of transgenic breeding required to deliver the required numbers. We estimate that a maximum of 700 per year might be used in infection protocols if our vaccine projects are active however this is an upper estimate and the actual number used is likely to be very much less.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our experiments incorporate longitudinal monitoring (monitoring of the same mice over a period of time) thereby greatly reducing the number that need to be infected at individual time points by 5-fold, depending on the number of time points.

We also try to identify a suitable fixed single time point to end an experiment; this ensures that we obtain as much quantitative (numeric) data as possible from that final, planned time point. This has largely replaced the practice of waiting for a particular symptom to develop, as the practice is too unpredictable and does not take animal welfare into consideration.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will undertake pilot experiments using a very small number of mice on each occasion where there is a new variable, for example, a new bacterial strain type, as the effect of the new strain will be unknown. This information will be used to undertake statistical tests to ensure the optimum number of mice are used per group, wherever practical.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use intranasal models of infection, and soft tissue models of infection. Some mice will receive treatments by injection, or will receive vaccinations before infection. The models used have been scientifically validated and shown to provide valuable information while emulating the disease features of human infections. The duration of infection, and dosing regimens in these models has been adjusted and improved upon over several years to avoid severe outcomes and to cause least distress.

Why can’t you use animals that are less sentient?

In order to reproduce a mature mammalian adaptive immune response to infection or vaccination, it is necessary to use mature (adult) mice.

We cannot use species that are less sentient, such as insects or fish, because these do not provide the context required to model bacterial infection in humans i.e. being able to breathe air, with a mature adaptive and innate immune response.

For a minority of infections we will use terminal anaesthesia and thus animals are not aware of the procedures.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For any model where there is a risk of systemic illness, the frequency of monitoring will be increased. Where there is a risk of pain, analgesia will be provided in advance, as advised by our veterinary officer.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to guidelines issued by LASA and NC3Rs. Furthermore, we will endeavour to report our findings accurately using ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Institutional seminars and workshops will provide continuing professional development in the 3Rs, while the NC3Rs website provides a readily available resource at all times.

130. Synaptic and circuit mechanisms for neural computation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Neuron, Brain, Cognition, Navigation, Autism

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	embryo, neonate, juvenile, adult,pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our first aim is to understand how processing and storage of information by nerve cells contributes to cognitive functions of the brain. Our second aim is to determine how this changes in models of neurological and psychiatric disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.
Why is it important to undertake this work?

Understanding the biological basis of human and animal behaviour will help to answer centuries old questions about the physical nature of mental processes and may be of general importance for human mental health and disorders of the nervous system, such as depression, anxiety, schizophrenia and memory loss. The general importance of the work in this project will be three fold. First, new fundamental knowledge about how the cells in the brain carry out computations that underlie core cognitive processes will answer long-standing questions about how brains work. Second, new technologies developed during the project will further accelerate progress in understanding brain functions and in investigation of models of brain disorders. Third, advances made during the project may form a basis for new rational therapeutic approaches for brain disorders for which there are at present few effective treatments.

What outputs do you think you will see at the end of this project?

We hope to produce the following outputs:

New knowledge of the connectivity between nerve cells in brain structures important for cognition.

Advances in understanding how information is processed and stored in the brain.
New insights into how new experiences and stored memories influence behaviour.
New technologies that accelerate progress in understanding the brain.
An improved understanding of how changes in information processing and storage lead to symptoms of psychiatric and neurological disorders.

Who or what will benefit from these outputs, and how?

There is a global research effort to understand the workings of the brain and how it goes awry in disorders. The outputs will contribute directly to the effort.

In the medium term we hope that a key beneficiary will be patient populations through development of new therapeutic approaches.

A further potential group of beneficiaries are computer scientists aiming to develop brain like artificial intelligence algorithms.

How will you look to maximise the outputs of this work?

We aim to publish our work in widely read and well respected open access research journals. We will communicate with members of the scientific community at relevant conferences.

We plan to continue collaborations with clinical colleagues to translate our findings to human **patient populations.**

We intend to continue and extend ongoing industrial collaborations to see commercial impacts of our research.

Species and numbers of animals expected to be used

- Mice: 10000
- Rats: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use mice and rats. We use these animals as models for several reasons. First, the core cognitive functions we wish to understand and their underlying neural circuitry are well conserved across mammalian species. Therefore, while details may differ in humans, the general operating principles are likely to be similar. By making advances first in rodents, it is then much easier to address whether similar mechanisms arise in the human brain. Second, while similar questions could be addressed in primates, we believe that it is ethically preferable to use less sentient species for experimentation where possible. Third, it is not possible to use simpler established models, for example flies or worms, for the questions we aim to address as these species do not employ the same cognitive functions. Fourth, a remarkable array of experimental tools is available for neuroscience experiments with mice and rats. This enables more rapid progress than would be possible using other similar species.

The project will primarily focus on mature animals as it is cognitive functions in mature animals that we wish to understand. Where development is relevant we may also investigate younger animals, for example when translating our findings to models of developmental disorders, for example autism spectrum disorders.

Typically, what will be done to an animal used in your project?

The project will typically involve two different types of experiment:

To investigate the organisation of connections between neurons, a typical experiment will involve introduction of biomarkers into particular populations of neurons, humanely culling the animal followed by removing the brain from the cadaver and then visualising the connections or assessing functionally how one population of neurons influences other populations.

This experiment requires surgical procedures for injection of biomarkers to targeted brain areas. These procedures are carried out under anesthesia and typically have duration less than 60 minutes.

To investigate information processing and storage by neurons, a typical experiment will involve recording and manipulation of the activity of neurons as animals perform cognitive tests. The recordings of neural activity will tell us what aspects of the test and its solution the neuron represents. The manipulations will enable us to test contributions of neurons to performance on the test. This experiment requires surgical procedures to implant electrodes or optical devices into the animal's brain. The electrodes or optical devices are then used to readout activity of nerve cells in the brain.
The procedures for their implantation are carried out under anesthesia and typically have duration less than 90 minutes.

What are the expected impacts and/or adverse effects for the animals during your project?
The major expected impacts arise from experiments requiring injections of substance into the brain and implantation of devices for recording from the brain. These may include transient pain and weight loss, which will be mitigated with pain relieving medication and supportive care. These procedures can be carried out with refined surgical protocols and are not typically associated with other adverse effects.
They are not expected to cause distress or suffering to the animals.

Animals may be housed singly where there is a concern of aggressive behaviour or increased likelihood of damage to implanted devices, for example for electrophysiology or optical experiments. Single housing is thought to be stressful to rodents and will only occur when scientifically justified. In such cases, the duration will be limited to the minimum period necessary and all cages will be enriched with objects for exploration and play. These methods have been shown to reduce stress in rodents.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity is not expected to be classified above a moderate level. This applies to all animals used for the project. We expect that for 80% animals the severity will be mild or subthreshold since they are only used in breeding and humane killing for tissues, while 20% will experience moderate suffering on the experimental (surgical) protocols.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We want to understand how cells in the brain mediate its cognitive functions. Because these functions of the brain rely on sensory input and manifest as outputs that change behaviour, we can only study how they work using live animals that are able to carry our measurable behaviours.

Which non-animal alternatives did you consider for use in this project?

Computational models can be used to simulate aspects of brain function and to make predictions about how biological substrates can implement cognitive functions.
Cell culture systems can be used to study molecular mechanisms and certain aspects of communication between neurons.

Why were they not suitable?

Computational models are useful as part of an overall refined approach, but it is not possible to use them as alternatives to answer the questions we aim to address. Essentially, the models don't yet explain the cognitive functions we wish to understand. Development of better models requires data that can only be obtained with experiments in which animals use the cognitive functions we aim to understand.

Cell culture systems do not have the organisation of real brains, do not process sensory inputs and do not execute behaviours. They therefore can not be used as alternatives to answer the questions we aim to address.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate of animal numbers takes account of the experimental goals for the period of the project, the number of animals required for experimentation and the numbers required for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We aim to design experiments that test clear hypotheses and are likely to have large effect sizes and therefore conclusive outcomes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will carefully monitor breeding of animals**.**

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice and rats. We will use methods for investigation of connections between nerves cells, and for recording and manipulation of neurons in animals carrying out behavioural tests.

Why can’t you use animals that are less sentient?

We want to understand cognitive functions such as episodic memory, navigation and decision making. These functions are either absent in less sentient animals or are implemented through mechanisms that diverge substantially from those used by mammals including humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will adopt a number of refinements that minimise welfare costs, which by increasing the precision of experimental tests and manipulations will lead to clearer and increasingly specific answers to the questions we aim to address. These will often involve adopting specific molecular and ex-vivo strategies for precisely targeting single nerve cells or populations of nerve cells instead of invasive and less specific in vivo methods. We will also adopt behavioural assays that give precise control of the experimental animal’s sensory experience and clear read outs of cognitive outputs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow all best practice guidance provided by our institution. We will also follow procedures that we and others have published that establish refined methods for the specific experimental questions we aim to address.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are fortunate to be able to rely on excellent institutional support and training to stay informed about and implement advances in the 3Rs. The Home Office Liason Contact (HOLC) circulates HO guidance notes and welfare organization’s newsletters through emails. The Named Veterinary Surgeon (NVS) team and full time Named Training and Competency Officer (NTCO) work together with PIs to refine procedures and disseminate best practice ideas.

131. Improving prognosis from vascular dementia

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words
vascular cognitive impairment, mice, behaviour, vascular dementia, endothelial inflammation

Animal Types	Life Stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this research project is to improve our understanding of the pathophysiology of vascular dementia in order to facilitate the development of potential treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Vascular dementia is a pressing concern as there are over 150 000 people currently living with vascular dementia in the UK, but also many other types of dementia (like Alzheimer’s disease) co-exist with vascular dementia. Dementia is a global health challenge as the incidence is doubling every 120 years. There are no effective treatments for vascular dementia. Therefore, there is an extreme need to better understand how it develops and to design interventions that can help patients.

What outputs do you think you will see at the end of this project?

The main output of this work will be information. Information will be disseminated at national and international scientific meetings. Information will also be disseminated via science communications and social media. We aim to publish new information in scientific journals with open access policies so information will be accessible to the public.

Who or what will benefit from these outputs, and how?

There will be immediate benefit for the students and fellows that will gain important knowledge and this will continue as they use their skills to continue future work. There will be benefit to the wider scientific community in the mid-term via best practice recommendations surrounding the establishment of new cognitive testing strategies for animal models of vascular dementia. There will also be mid to long term benefit as the information produced will improve our understanding of the pathophysiology of vascular dementia. This knowledge will enable the development of treatment options that could result in benefit to patients and medical practitioners long term.

How will you look to maximise the outputs of this work?

We have successful collaborations with several laboratories that provide access to additional methods and analysis strategies that will allow us to examine data in novel ways. We also collaborate with neurologists that specialise in cerebrovascular research; this helps to focus the projects on tangible translational outcomes. In addition to regular publication of research findings, we have an active social media presence and engage with scientific communication to the public. I have a history of publication of unsuccessful approaches, despite the challenges associated with it.

Species and numbers of animals expected to be used

- Mice: Approximately 400 mice

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The adult mouse is the lowest sentient species suitable to model a progressive condition in which the primary symptom is a decline in cognitive function with age. Mice can be used for a large number of outcome measures, including behavioural testing, imaging and ex vivo tissue analysis. Mice are the only animals that allow for future work that may include genetic manipulation and this would be the next step to confirm new knowledge generated by this project.

Typically, what will be done to an animal used in your project?

Typically, mice will undergo behavioural assessments that mainly focus on testing memory. Most of the tests can be conducted within two weeks. Some mice may undergo

daily testing in an automated chamber that will test decision making and rule learning. These mice will undergo food OR water restriction to motivate performance. This would be the experience for approximately 20% of the mice, but weight will be monitored daily to ensure that animals do not drop below 85% of free feeding weight. Furthermore, the mice would have access to high value food rewards while performing this test.
Typically, mice would undergo a surgical procedure to reduce blood flow to the brain, or a sham surgical procedure. Both procedures involve an incision and implantation of restricting (or non- restricting in the case of sham) devices on both carotid arteries. The animals may lose up to 3% of body weight after surgery, and they would be monitored daily. Typical behavioural assessments would be repeated between 2 and 6 weeks post-surgery, following recovery. During the entire 2 month period, there may be an MRI scan. This would be the experience of approximately one third of the animals. The animals will typically be killed by perfusion fixation at the end of the study and tissue harvested for investigations. A typical mouse would undergo one surgical procedure and behavioural testing, and would be killed after 2 months.

What are the expected impacts and/or adverse effects for the animals during your project?

All animals that undergo food OR water restriction (these procedures will not be performed at the same time) will experience hunger or thirst and this is mildly adverse. Some animals may experience weight loss, but this will not be allowed to drop below 85% of free-feeding body weight. Mice will be without food for a maximum of 23 hours, or water for a maximum of 16 hours per day. The minimal amount of restriction required for motivation will be employed. To minimise stress, mice will be supplemented with extra food/water on the weekends and diet is supplemented with high value rewards in the automated chambers (some mice perform in excess of 200 nose poking trials). There are no other adverse effects associated with the other behavioural tests apart from mild stress. This will be controlled with appropriately handling and habituation. There is stress associated with swimming in the water maze.

The water is moderately adverse in order to motivate escape. Stress will be minimized by ensuring the water is of an appropriate temperature, and initially habituating the mice to the maze with a visible platform so they quickly learn how to escape. Animals will be hand dried and kept in proximity to a heat source to assist with drying.

All animals will experience moderate discomfort and pain at the incision site as a result of the surgical procedure. Analgesics will be provided to assist with post-operative pain relief. However, it is difficult to distinguish shams from hypoperfused animals. Hypoperfused animals generally lose no more than 3% of body weight, which recovers to baseline within 4 days. Condition will be recorded and monitored once per hour until recovery and at least 3 times in the first 24hrs. Animals will be humanely killed if they exhibit signs of poor health that would exceed the moderate severity banding.

All animals will experience moderate discomfort as a result of the anaesthetic procedure required for MRI. However, recovery generally occurs quickly (within an hour) and there are no signs of abnormal behaviour or distress. Nevertheless, scan times will not exceed 4 hours and there will be no more than 2 scans in the animals’ life time.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

All mice will experience moderate severity. The hypoperfusion surgical procedure requires anaesthetic and mice will experience pain surrounding the incision site following recovery. They may also experience mild weight loss (3%) and develop cognitive impairments with time. The mild stress associated with any accompanying behavioural tests will not exceed this moderate severity limit.
Approximately 20% of the mice will undergo food or water restriction to motivate performance in the operant conditioning chambers. The minimum amount of restriction required to produce motivation will be employed. Animals will be supplemented with food/water over weekends. This procedure will also not exceed moderate severity as there is no lasting pain or harm. The additional anaesthetic associated with an MRI scan (approximately 1/3 of animals) is also not anticipated to exceed the moderate severity banding. While there is some stress and anxiety associated with the anaesthetic, there is no pain or lasting harm and the mice generally recover very quickly.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is no suitable alternative to animals to model a chronic, progressive, and heterogeneous condition in which the primary symptom is a decline in cognitive function. We wish to assess complex behaviours, and this is not possible without animal models.

Which non-animal alternatives did you consider for use in this project?

We considered using in vitro preparations, for example, induction of inflammation in endothelial cell cultures. We have also considered mathematical modelling as an alternative to animals.

Why were they not suitable?

We use in vitro cell culture preparations routinely to examine cellular changes and molecular pathways. However, it remains impossible to extrapolate these findings into cognitive changes at the level of the whole organism. In vitro cell culture preparations will complement our in vivo approach, they just can not replace them completely. Mathematical models require some input, and there are too many unknown variables to predict. However, we are currently using mathematical modelling with some of our neuroimaging data to obtain a preliminary understanding of the changes in the brains of our animals in response to vascular risk factors. This information could be applied to future experiments as a possible replacement strategy.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

There are several strategies that can be used to approximate the number of animals required. One strategy involves comparison to existing literature, and group sizes tend to be in the range of 5-8 for this model. We have found that small group sizes tend to have higher variability and particularly for behavioural measures, group sizes of approximately 12 animals result in predictable variability.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have applied the principles of Good Laboratory Practice to design experiments. We have carefully considered the requirement of control groups, such as sham procedures to control for the effects of anaesthetic, surgical incisions, analgesics and pre or post-operative care on behavioural outcomes, or, on the vascular inflammatory responses we wish to measure. We have also considered the feasibility of using the same group of animals to address multiple objectives (ex vivo tissue samples or imaging to follow a cohort). We have made use of the NC3Rs Experimental Design Assistant (I collaborate with some members of the EDA working Group) and this tool will be used to help design study protocols.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have specifically requested the ability to perform essential pilot studies to help refine new outcome measures. We have also received mouse brain tissue samples from collaborators using this model to help shape some of the objectives of our experiments. For example: we have found evidence of inflammation in the endothelial cells from the brain sections.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Model:

The only available models of sporadic (environmental rather than genetic) vascular dementia are produced via hypoperfusion to the brain. These are considered refined as there are few adverse effects associated with the surgical procedure required to induce

brain hypoperfusion. Aside from some pain due to the incision site, and distress associated with anaesthetic, animals generally recover well following surgery. They are indistinguishable from shams and do not exhibit excessive weight loss.
They maintain the ability to feed, groom, move or climb. In mice, the most refined hypoperfusion procedure is accomplished via stenosis of the carotid arteries with small microcoils, as complete ligation can lead to severe outcomes.

Methods:
Behavioural testing:

The most refined methods for non-aversive handling will be used wherever possible throughout the experiments.

The most refined methods to examine executive function involve operant conditioning. The mice are offered high value food rewards in exchange for correct responses (nose poking into light cued holes). Food OR water restriction is necessary as high value rewards are not sufficient to induce poking responses in all mice. Food restriction represents the most refined method of motivation as hunger causes the least amount of distress. The animals will be without food for a minimum of 4 hours and up to a maximum of 23 hours per day for a maximum of 12 consecutive weeks. We aim to refine this by employing the minimal amount of restriction time required to motivate the animals to perform in the behavioural test. Weight loss will not reduce to less than 85% of free-feeding body weight and animals will be returned to free-feeding status if weight can't be maintained at appropriate levels. Water restriction represents the next most refined method to motivate performance and is preferred in situations in which dominant animals may hoard food. The animals will be without water for 12-16 hours prior to testing for a maximum of 5 consecutive days. We aim to refine this by employing the minimal amount of restriction time required to motivate the animals to perform in the behavioural test.

Weight loss will not reduce to less than 85% of free-feeding body weight and animals will be returned to free water status if weight can't be maintained at appropriate levels. Both restriction regimes have been published extensively, and while there is evidence that water restriction results in better performance in operant conditioning tasks, the discomfort scores are slightly higher than those when mice are food restricted. However, discomfort scores under both regimes were categorised as mild (Goltstein, et al., 2018. PLoS ONE. 13: e0204066).

The Y, radial arm and water maze are the most well characterized, widely published tests to benchmark spatial learning and memory. They are considered the most refined, as deficits are predictable with a minimal amount of distress for the mice.

The water maze is the only test that is associated with distress that can't be alleviated with handling and habituation. The animals will find the water uncomfortable (it is the aversive stimulus required to motivate escape). To minimise distress, the water will be warmed to 24 ± 2°C and mice will not undergo more than 4 trials per day (no more than 90s per trial) and there will be an inter-trial interval of at least 20 minutes. Animals will be hand dried with paper towels and kept in proximity to a heat source to assist with drying.

Cognitive tests that do not require spatial navigation may also be employed to assess short term memory. Novel object recognition allows animals to explore an environment with new objects. This test is considered the most refined test of object memory as distress or discomfort is unlikely.

Mice may also undergo simple sensorimotor tests to ensure cognitive deficits are not due to underlying sensorimotor problems. The rotarod is the most refined as it is well characterized and widely published whilst only being associated with a mild stress for the animals due to the short fall.

Magnetic resonance imaging:

MRI can be considered the most refined method to measure various aspects of brain structure and function, as it is completely non-invasive. The distress that is expected will be due to the anaesthetic, but there will be no pain or lasting harm.

Why can’t you use animals that are less sentient?

We have selected the mouse as the lowest sentient species suitable for replicating cognitive behaviours that occur as a result of the interaction between the vasculature and the brain. Mice can be used for a large number of outcome measures, including behaviour, imaging, and ex vivo tissue analysis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Model:

To minimise harms for the animals following surgery, pain relief (analgesics) will be provided with consultation from the NVS. All animals will be provided with wet diet to assist with feeding and appropriate fluid replacement, until weight stabilises. Animals will be monitored once per hour until

recovery and at least 3 times in the first 24hrs. In the following days, animals will be monitored at least twice a day until weight stabilises.

Behavioural testing:

To minimize the discomfort associated with food restriction, animals will be maintained at 85% of initial free feeding weight (measured daily) and the shortest possible restriction times that still result in motivation will be used. Typical water restriction schedules allow 1hr of unlimited access to water within every 24hr period, but we will use the shortest restriction schedule possible to still motivate poking responses. Weight will be monitored daily.

Stress will be minimized in the water maze by maintaining the water temperature above 20°C (24 ± 2°C
). Stress can also be reduced by initially habituating the mice to a visible platform so they quickly learn how to escape. Limiting trial times will minimize fatigue and mice will be dried and kept in proximity to a heat source before being returned to their cages.

Magnetic resonance imaging:

The distress associated with MRI will be minimized by restricting the number of measurements and measurement time (4hours), for each animal. Animals will not undergo more than 2 imaging sessions over the course of their lifetime, and there will be a

minimum 24hrs between sessions in order to allow adequate recovery time from the anaesthesia.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Good Laboratory Practice, the ARRIVE guidelines, and relevant NC3Rs guidelines to ensure experiments are conducted in the most refined way.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We regularly monitor the 3Rs webpage and Twitter feed, and follow all communications. If advancements that could help refine our models occur during the course of the study, we will be enthusiastic about implementation.

132. Evaluation of cancer therapies in orthotopic cancer models

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunology, Orthotopic, Therapy, Vaccine

Animal types	Life stages
Mice	Adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to evaluate identified cancer therapeutics which can be used to prevent or treat orthotopically implanted cancers.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite significant progress, cancer continues to be a major cause of mortality in humans with the lifetime risk of an individual developing cancer in one form or another being approximately 1 in 2. Approximately 440 people die of cancer every day in the UK (Cancer Research UK statistics).

Although current treatments such as radiotherapy, chemotherapy and hormone treatments can be effective against primary and secondary cancers, they can be associated with significant toxic side- effects, and therapeutic resistance often occurs. The spread of primary disease to other tissues (metastasis), tumour relapse and resistance to therapy remain the principal causes of death for patients with cancer. Metastasis is a particular problem and accounts for ~90% of cancer-related deaths.

What outputs do you think you will see at the end of this project?

The outputs of this project will include;

- Publications in Open Access Journals.
- Dissemination of information at National and International Conferences.
- Identify a treatment approach that could progress to a clinical trial.

Who or what will benefit from these outputs, and how?

The immediate benefit from this project will be for basic science by producing world-leading and internationally recognised work that will be published in high impact journals.

Output from this project may be utilised by others including preclinical academics, industrial partners and clinicians. We will disseminate our results via both National and International conferences and workshops.

While Basic science will benefit in the short-term (1-3 years), clinical impact may take a longer period to be achieved. This may have the potential to be a therapeutic or diagnostic tool.

The time taken to fully evaluate a given therapeutic or diagnostic tool is likely to go beyond the five-year duration of the programme of work due to the complexities involved in the research.

How will you look to maximise the outputs of this work?

The collaborations and services provided will lead to both the furthering of our existing knowledge and build new knowledge surrounding treatments in orthotopically implanted cancer. This may lead to joint applications for funding if the results show promise.

It is anticipated that the findings from these outputs may be disseminated through peer-reviewed journals, conferences and marketing materials.

Species and numbers of animals expected to be used

- Mice: 500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All of our experimental procedures will be performed in either wild-type or genetically-altered mice models. All the studies in this project will use adult mice from 6 weeks of age.

Mouse models have many similarities to humans in terms of anatomy, physiology and genetics in the way tumours metastasise. The animals used in our research will help us understand the mechanisms that underpin cancer, such as the growth and spread of tumours, and to develop new ways of diagnosing, treating and preventing the disease.

Mouse models are essential in cancer research. They are used to understand the genetic basis of tumour development and cancer progression. The MHC transgenic mouse models we propose to use have the same HLA haplotypes as humans for Class I and Class II which makes the results obtained more translatable in humans. They can also be used to test the efficacies of different anti-cancer agents. Moreover, in translational cancer research, they represent a powerful tool in assessing the potential validity of targeted therapy because the targets can be precisely studied in the setting of a developing or developed tumour.

Typically, what will be done to an animal used in your project?

Typically actions in this project include:

Implantation of tumour cells orthotopically

Administration of substances

In vivo imaging Blood sampling

Animals will be humanely killed at the end of the experiment

The animals will be implanted with tumour cell lines orthotopically to establish the appropriate tumour growth rate. Blood samples may be taken from a superficial vessel under terminal anaesthesia. Imaging may be performed up to three times a week in which case the animals may be injected with substrate prior to induction of anaesthesia and placement into the in- vivo imager.

The animals in this project may be injected on more than once occasion with test items (by one or more of the following routes -subcutaneous, intravenous, intraperitoneal, intradermal, intramuscular, intranasal, gene gun, gavage, topical, drinking water, diet or infusion via a pump or pre-implanted cannula(e) attached to a subcutaneously implanted mini-pump.

The typical duration of these types of studies will vary from between 4 weeks to 12 weeks and beyond dependent on the design of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that animals may experience moderate discomfort as a result of tumour cell implantation and the pump or pre-implanted cannula(e) if fitted. Some animals will experience mild transient discomfort during this project as a result of administration of substances, blood withdrawal and in vivo imaging.

A bodyweight loss of up to 18% may be observed up to 5 days post-surgery, however, recovery would be expected after this time.

Following 5 days post-surgery if the onset of clinical signs such as lethargy, ataxia is observed, the animals will be monitored on a more frequent basis, (at least 3 times a day). If weight loss of up to 15% in combination with other clinical signs is observed, which would indicate a deterioration in the physical condition of the animal, and it is at this point the animal will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

It is inevitable that a small proportion of animals will show adverse effects, due to the nature of the intervention.

The severity level of this project is moderate and it is anticipated that approximately 94% of the animals used will fall under this severity level.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although this research employs an extensive portfolio of in vitro laboratory techniques, these cannot adequately model the complete array of biological and immunological events that are involved in tumour growth and microenvironment or the generation of protective anti-tumour immunity, all of which are important to our understanding of tumour

immunology and the development of new therapeutic strategies in orthotopically implanted tumours. In -vivo studies in mouse models are therefore essential as they have the integrated physiological systems which are required.

Which non-animal alternatives did you consider for use in this project?

2D and 3D cell cultures can be used to some extent to assess the effect of a given treatment on a tumour cell line in vitro, however, they cannot completely replace the information gained from animal experiments as the entire tumour microenvironment is difficult to replicate in the laboratory.

Why were they not suitable?

In -vitro studies can produce valuable information, however, they cannot mimic the exact in -vivo environment to exhibit the effects that a whole biological system has on disease progression and therapeutic intervention.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals have been estimated based on previous usage and expected usage (up to 30 animals per study) based on conversations with researchers and service users.

In-house discussions with a biostatistician were held to review our experimental designs and the numbers are also based on our previous usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculation studies. We will also apply the NC3Rs experimental design assistant tool for appropriate experimental planning. We will regularly consult qualified statisticians about experimental design and perform imaging and histology for tumour evaluation where possible. We will regularly consult with the qualified statisticians about experimental design and statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Both sexes of animals may be used in the experiments.

Double transgenic (2 genetic modifications) will be used whenever possible, thereby reducing the number of animals being used.

Pilot orthotopic tumour growth studies will be performed where appropriate to establish tumour growth rates prior to use in the prophylactic and / or therapeutic studies.

All experiments using live animals will adhere to the ARRIVE guidelines on design and reporting. Good principles of experimental design will be applied to ensure sufficient group sizes will be used to adequately test the hypothesis. Sample sizes are estimated from pilot studies and previous data using power analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically-altered mice bred and maintained are of a non-harmful phenotype. Prior to implantation all cells are prepared in a sterile environment and are administered using sterile technique.

Cells will be administered orthotopically in a sterile manner under general anaesthesia. Animals will receive analgesia pre and post-surgery in order to mitigate pain and discomfort.

Substances are prepared and administered using sterile techniques. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be in accordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

Pilot studies will inform on tumour development and end-points prior to moving into a therapeutic setting and these will be used to determine the most appropriate method of assessment of tumour development. It is anticipated that some animals will be monitored using body weight measurements and some animals will be monitored via imaging which will require transient anaesthesia to immobilise them while an image is acquired. Once completed, the animals are expected to make a full recovery within 30 minutes. Each method will allow us to identify tumour growth rates and end-points.

Blood sampling will be performed using sterile techniques and volumes collected will be in accordance with the LASA / NC3Rs guidelines. We will aim to take the smallest volume which will allow for adequate analysis.

All animals will be humanely killed a Schedule 1 method.

Why can't you use animals that are less sentient?

The studies proposed in this project could not be undertaken in animals with a lower form of immune system (e.g. drosophila, C.elegans) because those models do not show comparable responses that are seen in humans. Furthermore, to achieve the maximum relevance and utility from this project, it is essential that we use genetically altered transgenic mouse models e.g., for particular human HLA types as these have been used previously and identified areas of interest which will be investigated further under this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over the last 5 years, a number of refinements have been made and considerations to refinements is an ongoing process. The procedures that are in place to administer substances and monitor the animals will be reviewed regularly and techniques will be refined where possible and additional monitoring is performed where necessary and is carried out by the PIL holders. Any relevant refinements made are discussed and disseminated to the other users by the animal care scientists.

The Veterinary surgeon also observes the work undertaken under this project and will offer suggestions for refinements where necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by the Workman Group on welfare and use of animals in cancer research published in 2010. The ARRIVE guidelines Animal Research: Reporting of In Vivo Experiments) and LASA Good Practice Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain close interactions with the relevant welfare, training and information officers as they oversee and perform in vivo studies. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and newsletters which are circulated monthly.

Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

133. Production of antibodies, antisera and blood products iii

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Blood products, Tissue samples, In-vitro diagnostic reagents, Controlled environment, Antibodies

Animal types	Life stages
Rabbits	adult, juvenile
Llamas and Alpacas	juvenile, adult
Pigs	juvenile, adult
Animal types	Life stages
Goats	juvenile, adult
Sheep	juvenile, adult
Cattle	juvenile, adult
Guinea pigs	juvenile, adult
Mice	juvenile, adult
Rats	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This service provided under this project licence will supply in-vitro diagnostic reagents and animal tissues to research groups and biomedical companies, nationally or internationally to further their fundamental and applied research for the development and application of research projects and new treatments in support of human and animal healthcare.

This includes:

- Provision of blood products Provision of tissue samples
- Production of antibodies to microorganisms e.g. bacterial / viral antigens for use in diagnostic tests
    - Production of antibodies to purified peptides / proteins

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This service provided under this project licence will supply in-vitro diagnostic reagents and tissues for ex-vivo studies to laboratories, nationally or internationally and underpins research ranging from fundamental or specific disease focused basic research to the commercial development of new treatments in support of human and animal healthcare. Antibodies play a part in many aspects of today's drug discovery and development. The antibodies can be then used in a multitude of applications, including but not limited to western blot (WB), immunoprecipitation (IP), immunofluorescence (IF), immunohistochemistry (IHC), chromatin immunoprecipitation (ChIP), and flow cytometry (FC). As a cornerstone of the body's immune response, antibodies can provide significant data to support scientists' research.

Drug discovery starts with identification and validation of targets, and antibodies are the gold standard when it comes to the specific detection of an interesting biomolecule or pathway. Most life science laboratories use antibodies in some way. Due to their outstanding specificity they make exquisite tools that allow researchers to identify molecules that cannot otherwise be identified. Thus enabling conclusions to be drawn about the target molecule and pathway of interest. In addition, the management of infections, in humans and animals, due to microorganisms is aided by the use of appropriate antibody diagnostic tests. Polyclonal antibodies are invaluable tools for research and diagnostics. The main reason for this is their ability to provide signal amplification. These antibodies can bind to several epitopes of the same antigen. Thus, several antibodies can bind to the same target antigen, leading to a strong signal or more effective capture of the target antigen. The use of antibodies in research has been the cornerstone of numerous discoveries and they will continue for the foreseeable future. The use of whole blood and its products is a key step in large numbers of research projects. Be this as a source of immune cells, all major immune cells can be found in the most natural condition in freshly drawn peripheral blood. In addition, peripheral blood is increasingly used to obtain stems cells. Or as a reagent within a particular in-vitro test. For example for:

- Addition to microbiological media to grow fastidious microorganisms Production of standard reagents for laboratory procedures or diagnosis

- Developing techniques for determination of immunological response (e.g. ELISA or RIA) Normal negative controls for diagnostic assays
- Dilution of antisera for diagnostic assay kits

This licence will provide whole blood and its products to aid research and development projects. Where possible, this will be obtained from other sources (e.g. abattoir, butcher). On occasions the use of abattoir blood isn't appropriate. For example, if it is not possible to guarantee the sterility of blood obtained from an abattoir. As it is not possible to sterilise whole blood products, blood must be collected aseptically from a live animal.

Isolated tissue and organ preparations allow researchers to investigate the physiology and pharmacology of various tissue samples in a controlled environment without the complications of an intact animal model. These in vitro/ex vivo experiments can be performed using a variety of tissues and organs including smooth muscle, skeletal muscle, cardiac muscle, gastrointestinal and urogenital tissue samples. Because of the high metabolic activity of certain tissues, especially hearts it is not possible to obtain these tissues from other sources or after the animal has been killed using a schedule 1 method, due to the prolonged times and crude extraction procedures of non-research facility removal protocols.

For hearts it is necessary for the explantation process to be carried out under controlled conditions by trained personnel with immediate cardioplegic arrest (minutes), in order for the samples to remain alive and viable for the ex vivo studies. These tissue samples must be removed under terminal anaesthesia.

What outputs do you think you will see at the end of this project?

Research undertaken using tissue samples or antibodies supplied via this project licence will be used to advance the science of the respective projects.

This will either be by the progression of the strongest candidates into clinical development with the intention of developing marketable products or the development of new processes or equipment to aid recovery or quality of life of patients.

They will lead to an increase in the understanding of the underlining biology behind the diseases investigated.

The data generated from these studies will be used to help the researcher understand the biology behind the diseases of interests. Data will also be used for filing new patents and thus disseminated through the patent publication pathways.

In addition, to patent applications, scientific publications and conference presentations will be used to disseminate key scientific findings and promote the general advancement of the research studied.

Who or what will benefit from these outputs, and how?

In the first instance the tissue samples and antibodies generated from studies will aid the researchers in their continued research. Allowing for the continued research for new treatments for diseases which will lead to their further development (e.g. in clinical trials) and could potentially lead to new therapies being introduced to the market.
Long term, the research projects have the potential to significantly enhance the quality of life for people and animals suffering from diseases or potentially cure them. This will

benefit the whole society via reduction in absenteeism from work or school and reduction in demand on health services.

How will you look to maximise the outputs of this work?
Our commercial client will, where not confidential, look to publish the information via scientific publications and conference presentations in addition to patent applications. One of the key goals of our academic clients will be to p**ublish the results via scientific publications and conference presentations, in order to promote the general advancement of the fields studied.**

Species and numbers of animals expected to be used

- Cattle: 300
- Sheep: 375
- Goats: 135
- Pigs: 450
- Camelids: 60
- Mice: 350
- Rats: 350
- Rabbits: 110
- Guinea pigs: 275
- Turkeys: 150
- Chickens: 150

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All animals will be juvenile or adult. These two categories are included as the immune system changes during development and specific ages of animals maybe required. This species included in this licence are: Camelid, Cattle, Pig, Sheep, Goat, Poultry, Guinea pig, Rabbit, Rat and Mouse.

In the first instance the species selected are the most relevant to the scientific aims of the particular project. Each individual requestor will be required to justify their species of choice as part of the formal request. These requests are often very specific in the nature of the samples or antibodies required and they are not available commercially. We are able to meet these very specific needs.

While we may use multiple species in a number of the protocols, we predict that pigs will be the species used the most to supply tissue samples. For example, pig hearts are very similar to humans and therefore the pig would be the species of choice for this research area. Because of the high metabolic activity of certain tissues, especially hearts, it is not possible to obtain these tissues from other sources. This is due to the prolonged times it takes to collect these samples and the complicated process required to successfully remove the organs.

For antibody production, camelids, sheep and goats will be the species used most often. Camelids produce specific antibodies called VHH fragments. The unique size and structure of these antibodies mean they are able to bind to hidden antigens that are not accessible to whole antibodies, for example the active sites of enzymes. Sheep and goats' antibodies have a high affinity and sensitivity, and are particularly adapted to small antigens or small epitopes. In addition sheep and goats allow the production of larger volumes compared to smaller species and the animal does not necessarily need to be culled as part of the process.

Typically, what will be done to an animal used in your project?

After arrival all animals **will be allowed at least 7 days to become acclimatised to the unit. Then depending on the protocol:**

Blood sampling: One or more blood samples will be taken. The animal will be assessed between each sampling and only sampled if considered to be fit and hea**lthy. At the end of the blood sampling period the animal will either be re-used for tissue sample collection, returned to stock or re-homed.**

Tissue collection: The animal will be terminally anesthetised and the tissue sample collected. After which the anim**al, still under terminal anaesthesia, is euthanised.**
Antibody production: The animal will be immunised with the antigen, given a booster injection if required and blood samples taken for antibody extraction. The animal may be used in one or more rounds of **antibody production. The animal will be assessed between each round and only used if considered to be fit and healthy. At the end, the animal will either be re-used for tissue sample collection, returned to stock or re-homed.**

What are the expected impacts and/or adverse effects for the animals during your project?

The provision of tissue samples should have no adverse effects on the animals. The collection will either be a simple blood sample or collection under terminal anaesthesia.

Antibody production should also have little or no impact on the animals.
This will involve simple injections and blood sample collection.
There is the potential for an animal to have an allergic reaction to the antigen which may induce some adverse effects.
This is expected to be transient and have no lasting effect on the animal.

Expected severity categories and the proportion of animals in each category, per species.
What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of animals, the severity level will be mild. However, as stated above, in some studies the animals may experience some adverse effects. These would only cause the animal a moderate level of distress which will in most cases be transient.

What will happen to animals at the end of this project?

- Killed

- Kept alive
- Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.
Why do you need to use animals to achieve the aim of your project?

At a certain point in research projects there is a point where in vitro studies cannot completely answer the question being asked. This is due to the **complexities of the microenvironment of tissues and the involvement of the immune system. This cannot be fully replicated in a laboratory setting.**

Because of the high metabolic activity of certain tissues, especially hearts, it is not possible to obtain these tissues from other sources or after the animal has been killed using a schedule 1 method, due to the prolonged times and crude extraction procedures of non-research facility removal protocols. For hearts It is necessary for the explantation process to be carried out under controlled conditions by trained personnel with immediate cardioplegic arrest (minutes), in order for the samples to remain alive and viable for the ex vivo studies. These tissue samples have to be removed under terminal anaesthesia. Blood is required as a source of primary cells for in-vitro/ex-vivo studies. Wherever possible, blood products will be obtained from abattoirs or after animals have been put down for other reasons. In many cases, abattoir samples may not be suitable due to contamination or lack of information and/or traceability on the specific animal being used. Also, it is not possible to sterilise whole blood products, blood must be collected aseptically from a live animal.

It is also now possible to generate antibodies using non-animal derived methods, thanks to developments of new technologies. There are however still cases where there are simply no non- animal derived antibodies available as a suitable alternative. Particularly in the generation of polyclonal antibodies. This is the area of antibody production that we focus on.

Which non-animal alternatives did you consider for use in this project?

The very nature of the blood and tissue sample supply included in this project licence means that at the point we are approached, the use of non-animal alternatives has been completed or cannot answer the questions being asked.

Phage display library monoclonal antibody production is a valid alternative to using animals to produce monoclonal antibodies.

Why were they not suitable?

Currently the production of polyclonal using phage display libraries is not as effective as using this system to produce monoclonal antibodies. There is a need still to produce polyclonal antibodies, particularly in applications where their heterogenous nature is beneficial for recognition of multiple epitopes.

When monoclonal antibodies are required, phage display libraries are the leading technology to use for the production. However, there are limitations. they can produce

large amounts of specific antibodies but may be too specific to detect across a range of species. They are vulnerable to the change of epitope. Even a slight change in conformation may lead to dramatically reduced binding capacity.

Developing a monoclonal takes time and requires high technical skills.

There is still a role for polyclonal antibodies in research. Polyclonal antibodies are ideal reagents in diagnostic assays and hemagglutination reactions due to their ability to recognize different epitopes of a target molecule. This makes them more sensitive and able to detect antigens in very low concentrations. The best use of polyclonal antibodies is to detect unknown antigens. Polyclonal antibodies are used as a secondary antibody in immunoassays (e.g. ELISA, western blotting, microarray assays, immunohistochemistry, flow cytometry). Their role is to bind to different epitopes and amplify the signal, leading to better detection.

In addition, with immunohistochemistry the effects of the tissue fixation and processing on the epitope is unknown and highly variable, Polyclonal antibodies can be a better option because their multi-epitope binding allows for antigen recognition even if some of these epitopes are affected by changes in an antigen's structure or accessibility due to the processing of the tissue samples.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies.

Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

These numbers have been calculated on the numbers used under the previous 2 project licences. The number of possible projects we are aware of, either from our external clients or from academic grants that are being submitted by researchers over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To ensure we use minimal number of animals required to obtain meaningful and relevant data, we have extensively consulted available literature, attended experimental design and statistical courses, discussed with statisticians and NC3Rs staff and reviewed information provided by the NC3Rs. All requestors will be required to justify the number of animals required and if appropriate show how they came to this number.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In many cases, the numbers of animals required will be reduced by re-using animals for the mild procedures e.g. blood sampling. There is evidence that the immunisation of animals with multiple antigens at the same time give a good antibody response and the

separation/purification of the different antibodies is possible. The animal experience is no different when give one or a multiple antigen immunisation. This can significantly reduce the number of animals required.

When providing tissue samples, we try to coordinate it so that multiple tissues can be taken from the same animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In the first instance the species selected for antibody production and blood products supply are the most relevant to scientific and logistical (e.g. sample volume) requirements of the project and within the constraints of the licence.

Study protocols will be designed to ensure that any harmful effects resulting from any procedure will be detected early. For example, by including specific periods of observation when we know adverse effects are most likely to occur, e.g. the first 15 minutes post-dose. Having a dosing regime that allows time between each animal dosed, if an adverse effect occurs, we can intervene to stop other animals being affected.

Terminal tissue/organ sampling will be conducted under anaesthesia, so the animal feels no pain or suffering during the procedure.

Why can't you use animals that are less sentient?

The studies, tissue and blood sampling undertaken will be very species specific. The most appropriate ones for reach request will be used. All requests will have to justify why that particular species is required.

The process of whole body anaesthesia, for simple procedures like blood sampling, would be more stressful to the animal than the actual sampling.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The least invasive route of substance administration will be used. Along with the minimum number of injections to produce the required response and smallest needle gauge appropriate will be used if the blood sampling can be fulfilled, (e.g. minimum blood volume for the species fits with the scientific aims) with one or more species e.g. sheep or cattle,

then the species which is easier to handle (therefore the procedure will be less stressful for the animal) and in ready supply will be chosen.

All animals will receive appropriate operative care in terms of anaesthesia and pain management both during and after the procedure.

When appropriate, blood sampling maybe conducted on farms, thereby reducing the stress to the animal by transporting them and introducing them to a new environment prior to sampling.

In house expertise further enhances animal welfare, by providing close collaboration with dedicated animal care staff and veterinary consultants, and ready access to highly skilled advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the NC3Rs guidelines on the "Responsibility in the use of animals in bioscience research" and consult all the relevant references listed therein, e.g. NC3Rs Blood sampling resource. (Reference: NC3Rs/BBSRC/Defra/MRC/NERC/Royal Society/Wellcome Trust (2019) Responsibility in the use of animals in bioscience research: expectations of the major research councils and charitable funding bodies. London: NC3Rs).

For substance administration the LASA substance administration guidelines will be consulted (Reference: Administration of Substances to Laboratory Animals: Routes of Administration and Factors to Consider. J Am Assoc Lab Anim Sci. 2011 Sep; 50(5): 600–613).

Animals will continually be monitored for signs of pain and distress, especially post-challenge, by use of the grimace scale;
https://www.nc3rs.org.uk/sites/default/files/documents/Guidelines/MGS%20

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continuously monitor publications and the NC3Rs website for new and alternative models that could be implemented as part of this project. In addition, articles on advances in the 3Rs are regularly p**ublished on our internal Users News Forum and other relevant information is circulated by AWERB. Whenever possible we will implement these refinements into our studies.**

134. Investigating the immunology of cancer and the development of cancer therapeutics

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunology`, Therapy, Tumour, Vaccine

Animal types	Life stages
Mice	adult, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify cancer therapeutics which can be used to prevent or treat cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite significant progress, cancer continues to be a major cause of mortality in humans with the lifetime risk of an individual developing cancer in one form or another being approximately 1 in 2. Approximately 440 people die of cancer every day in the UK (Cancer Research UK statistics).

Although current treatments such as radiotherapy, chemotherapy and hormone treatments can be effective against primary and secondary cancers, they can be associated with significant toxic side- effects, and therapeutic resistance often occurs. The spread of primary disease to other tissues (metastasis), tumour relapse and resistance to therapy remain the principal causes of death for patients with cancer. Metastasis is a particular problem and accounts for ~90% of cancer-related deaths.

What outputs do you think you will see at the end of this project?

The outputs of this project will include;

Publications in Open Access Journals.

Dissemination of information at National and International Conferences.

Identify a treatment approach that could progress to a clinical trial.

Who or what will benefit from these outputs, and how?

The immediate benefit from this project will be for basic science by producing world-leading and internationally recognised work that will be published in high impact journals.

Output from this project may be utilised by others including preclinical academics, industrial partners and clinicians. We will disseminate our results via both National and International conferences and workshops.

While Basic science will benefit in the short-term (1-3 years), clinical impact may take a longer period to be achieved. This may have the potential to be a therapeutic or diagnostic tool.

The time taken to fully evaluate a given therapeutic or diagnostic tool is likely to go beyond the five-year duration of the programme of work due to the complexities involved in the research.

How will you look to maximise the outputs of this work?

The collaborations and services provided will lead to both the furthering of our existing knowledge and build new knowledge surrounding treatments in cancer. This may lead to joint applications for funding if the results show promise.

It is anticipated that the findings from these outputs may be disseminated through peer-reviewed journals, conferences and marketing materials.

Species and numbers of animals expected to be used

- Mice: 7,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All our experimental procedures will be performed in either wild-type or genetically altered mice. All the studies in this project will use adult mice from 6 weeks of age.

Mouse models have many similarities to humans in terms of anatomy, physiology and genetics, in the way tumours metastasise. The animals used in our research will help us understand the mechanisms that underpin cancer, such as the growth and spread of tumours, and to develop new ways of diagnosing, treating and preventing the disease.

Mouse models are essential in cancer research. They are used to understand the genetic basis of tumour development and cancer progression. The MHC transgenic mouse models we propose to use have the same HLA haplotypes as humans for Class I and Class II which makes the results obtained more translatable in humans. They can also be used to test the efficacies of different anti-cancer agents. Moreover, in translational cancer research, they represent a powerful tool in assessing the potential validity of targeted therapy because the targets can be precisely studied in the setting of a developing or developed tumour.

Typically, what will be done to an animal used in your project?

In this project, mice will be bred to have specific genetic modifications which are not expected to be harmful and will be bred using natural mating and maintained for use in this or other protocols.

Typically actions in this project include:

Implantation of tumour cells

Administration of therapies

In vivo imaging Blood sampling

Animals will be humanely killed at the end of the experiment

The animals in this project may be injected multiple times with the candidate items that will be tested (by one or more of the following routes -subcutaneous, intraveneous, intraperitoneal, intradermal, intramuscular, intranasal, gene gun, gavage, topical, drinking water, or diet to assess whether the the therapeutics are immunogenic. The subcutaneous tumour models will be set up and the candidate therapies tested using pre-determined vaccine strategies. In some studies, blood samples will be taken from a superficial vessel followed by a sample taken under terminal anaesthesia. A second injection of tumour cells may be given to assess memory responses.

The typical duration of a study will vary from between 4-12 weeks and beyond dependent on the study design.

What are the expected impacts and/or adverse effects for the animals during your project?

It is expected that some animals will experience minor transient discomfort during this project as a result of administration of substances, implantation of tumour cells, blood withdrawal and in vivo imaging. During the tumour growth phase some animals may show signs of tumour ulceration for up to 7 days with recovery. This will cause the animal moderate discomfort.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Based on our experience in the previous licence, we estimate that in this project 47% will be subthreshold, 43% will be mild and 9% will be moderate.

What will happen to animals at the end of this project?

Used in other projects
Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Although this research employs an extensive portfolio of in vitro laboratory techniques, these cannot adequately model the complete array of biological and immunological events that are involved in tumour growth or the generation of protective anti-tumour immunity, all of which are important to our understanding of tumour immunology and the development of new therapeutic strategies. In vivo studies in rodent models are therefore essential as they have the integrated physiological systems which are required.

Which non-animal alternatives did you consider for use in this project?

2D and 3D cell cultures can be used to some extent to assess the effect of a given treatment on a tumour cell line in vitro, however, they cannot completely replace the information gained from animal experiments as the entire tumour microenvironment is difficult to replicate in the laboratory.

Why were they not suitable?

In vitro studies can produce valuable information, however, they cannot mimic the exact in vivo environment to exhibit the effects that a whole biological system has on disease progression and therapeutic intervention.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on previous usage and expected usage based on conversations with researchers and service users.

In-house discussions with a biostatistician were held to review our experimental designs and the numbers are also based on our previous usage.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In-house discussions with a biostatistician were held to review our experimental designs.

We will ensure that we use the minimum number of animals required to answer the scientific question by performing power calculation studies. We will also apply the NC3Rs experimental design assistant tool for appropriate experimental planning. We will regularly consult qualified statisticians about experimental design and statistical analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding will be performed in a way to maximise efficiency, reduce surplus mice and ultilise both sexes in the experiments.

Both sexes of animals maybe used in experiments.

Candidate vaccines will be designed and assessed for their suitability with the use of in silico and in vitro techniques prior to moving them into the animal setting.

Double transgenic (2 genetic modifications) will be used whenever possible thereby reducing the number of animals being used.

Pilot tumour growth studies will be performed where appropriate to establish tumour growth rates prior to use in the prophylactic and / or therapeutic studies.

All experiments using live animals will adhere to the ARRIVE guidelines on design and reporting. Good principles of experimental design will be applied to ensure sufficient group sizes will be used to adequately test the hypothesis. Sample sizes are estimated from pilot studies and previous data using power analysis.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered mice bred and maintained are of a non-harmful phenotype.

Prior to implantation, all cells are prepared in a sterile environment and are administered using sterile techniques and the lowest volume of cells.

Substances are prepared and administered using sterile technique. The route of administration is via the least invasive method appropriate to the model. The volume of substances to be used will be in accordance with the Laboratory Animal Science Association (LASA) good practice guidelines.

The assessment of tumour development will be assessed on their known characteristics. Some animals will be monitored using callipers to assess tumour growth rates and some animals will be monitored using the in vivo imager which will require transient anaesthesia to immobilise them while an image is acquired. Once completed, the animals are expected to make a full recovery within 30 minutes.

Blood sampling will be performed using sterile techniques and the volumes collected will be in accordance with the LASA / NC3Rs guidelines. We will aim to take the smallest volume which will allow for adequate analysis.

All animals will be humanely killed by a Schedule 1 method.

Why can't you use animals that are less sentient?

The studies proposed in this project could not be undertaken in animals with a lower form of immune system (e.g., drosophila, C.elegans) because those models do not show comparable responses that are seen in humans. Furthermore to achieve the maximum relevance and utility from this project, it is essential that we use genetically altered transgenic mouse models (e.g., for particular human HLA types) as these have been used

previously and identified areas of interest which will be investigated further under this project.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Over the last 5 years a number of refinements e.g., reduction in the number of injections for a given therapy, changing to a less invasive route of administration, increased frequency of clinical observations has been made and considerations to refinements is an ongoing process. The procedures that are in place to administer substances and monitor the animals are 'fluid' whereby any opportunity to refine a technique or ensure additional monitoring is performed where necessary, which is carried out by the licensed scientists. Any relevant refinements made are discussed and disseminated to the other users by the animal care scientists.

The veterinary surgeon also observes the work undertaken under this project and will offer suggestions for refinements where necessary.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by the Workman Group on welfare and use of animals in cancer research published in 2010, The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and LASA Good Practice Guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will maintain close interactions with the relevant welfare, training and information officers as they oversee and perform in vivo studies. The PPL holder will stay informed of advances in the 3Rs by regularly checking the NC3Rs webpages (https://nc3rs.org.uk/the-3rs) and the newsletters which are circulated monthly.

Moreover, the PPL holder will attend appropriate seminars, symposiums and conferences deemed suitable.

135. Isolation and propagation of virus in eggs

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Influenza, Vaccine, Pandemic, Epidemic

Animal types	Life stages
Domestic fowl (Gallus gallus domesticus)	embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To isolate and propagate influenza viruses in embryonated hens' eggs.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Influenza viruses continue to threaten human and animal health. They are best controlled by vaccines and antiviral medicines.

The vast majority of influenza vaccines for humans are produced in hens' eggs and require the initial virus from which the vaccine is produced to have been isolated in embryonated hens' eggs and to have been exclusively propagated in hens' eggs. Hens' eggs are also

the most appropriate for examining the properties of certain influenza viruses of animals (e.g. poultry) that infect humans, and so pose a threat to public health and pose a pandemic threat. This project primarily covers the isolation and propagation of human and animal influenza viruses in embryonated hens' eggs and the analysis of the properties of these viruses.

What outputs do you think you will see at the end of this project?

The work entails the isolation of influenza viruses in hens' eggs and their detailed characterisation. These isolates can be further developed for the generation of egg-propagated candidate vaccine viruses that can be used by vaccine manufacturers for the production of influenza vaccines. This will be done as an ongoing process throughout the period of the project.

Assessing the properties of new influenza viruses of animals that infect humans allows for an assessment of the impact such viruses can have on human health, this includes the susceptibility of such viruses to antiviral medicines. The results of these analyses will be shared with the World Health Organisation (WHO). This will also be done as an ongoing process throughout the period of the project. Work will be published in peer-reviewed journals where appropriate.

Who or what will benefit from these outputs, and how?

Public health will benefit from the work.

The properties of viruses of animals that infect humans, zoonotic influenza viruses, will be shared with WHO for their risk assessments of the pandemic potential of such viruses, and these assessments lead to the subsequent prioritization of viruses for the generation of vaccines for pandemic preparedness purposes.

The isolation and propagation of egg isolates of human influenza viruses are the first steps on the path to the provision of the large majority of influenza vaccines. Selected viruses will be further developed as candidate vaccine viruses and, ultimately vaccine viruses for the production of human influenza vaccines.

Examining antiviral treatments and assessing the sensitivity of newly emerging viruses to antiviral treatment also benefits public health.

How will you look to maximise the outputs of this work?

This work will continue throughout the project. It is critical to select viruses for isolation and characterisation in hens' eggs on a timely ongoing basis as the influenza viruses evolve. This ensures that the most appropriate viruses are available for seasonal influenza vaccines and for vaccines developed for pandemic preparedness purposes.

Virus isolates deemed suitable for further development will be shared with other laboratories that carry this out. Updates on progress will be shared with WHO and vaccine manufacturers.

Assessment of the susceptibility of newly emerging influenza viruses to antiviral medicines is one of the key factors in the assessment of the risk posed by zoonotic influenza viruses to human health and is part of a process conducted by WHO -The Influenza Pandemic Risk assessment (see https://apps.who.int/iris/handle/10665/250130).

Species and numbers of animals expected to be used

- Domestic fowl (Gallus gallus domesticus): 28,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

At present the vast majority of influenza vaccines are produced in hens' eggs and are based on viruses exclusively propagated in hens' eggs. Alternative platforms, cell culture-based vaccines and recombinant vaccines, have been developed but these are not yet extensively used and the work here is to produce viruses for use in the egg-based vaccine platforms. Under these circumstances, it is not possible to develop an alternative to the isolation of viruses in hens' eggs and have an adequate vaccine supply to meet public health needs.

Isolation of viruses following amniotic inoculation is needed for most human influenza viruses. The efficiency of this process is much higher following inoculation of 14-day old embryos rather than, for example 10-day old embryos. The use of the older aged embryos is not possible to avoid without compromising the number of isolates recovered.

The examination of antiviral treatment in ovo provides a mid-point between examining the effects in tissue culture and in small animal models. This would be done on 9-day old to 11-day old embyronated eggs, but is included in this application to cover an experiment that might be required to proceed into the 14th day of embryonic development.

Typically, what will be done to an animal used in your project?

Embryonated hens' eggs of typically 14 to 16 days of embryonic development are to be inoculated in the amniotic cavity with virus with clinical samples or virus having been propagated in eggs or in tissue culture. The eggs will be incubated at 34o to 37o for typically 48 to 72 hours and then chilled to 4o prior to harvesting the propagated virus.

Embryonated hens' eggs of typically 10 to 11 days of embryonic development are to be inoculated in the allantoic cavity with virus with clinical samples or virus having been

propagated in eggs or in tissue culture. The eggs will be incubated at 34o to 37o for typically 48 to 72 hours and then chilled to 4o prior to harvesting the propagated virus.

In antiviral experiments 9-day old to 11-day old embryos will be used and the effects of possible antiviral medicines on virus replication will be examined for up to four days and then chilled to 4o.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the influenza viruses used cause no obvious adverse effects on the embryo. For those that do, like highly pathogenic avian influenza viruses that can cause zoonotic infections and may have pandemic potential, pilot studies will be carried out on small numbers of eggs of younger age (typically 9- to 11-days of embryonic development) to determine the mean death time, usually between 24 and 48 hours following inoculation, and in subsequent work eggs will be chilled at least 2 hours prior to the mean death time to reduce severity.

Amniotic inoculation is a difficult procedure and a proportion of eggs can die as a result of damage to the embryonic membranes. Allantoic inoculation causes very little damage to the embryo.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

As indicated above, most of the viruses used cause no obvious adverse effects on the embryo. Most highly pathogenic avian influenza viruses are likely to be inoculated into earlier aged embryos and so will fall outside of the scope of the act. Work on such viruses has represented less than 5% of the viruses with which we have worked in recent years. Inoculation with such viruses can kill the embryo however, this is prior to sentience (2-days prior to hatching) and so severity is deemed mild. Chilling at least two hours prior to death is used to reduce impact on the embryo.

Amniotic inoculation can result in the loss of up to 20% of the inoculated embryos. Allantoic inoculation results in the loss of approximately 5% of embryos.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As indicated above, at present the vast majority of influenza vaccines are produced in hens' eggs and are based on viruses exclusively propagated in hens' eggs. Alternative platforms, cell culture-based vaccines and recombinant vaccines, have been developed but these are not yet extensively used.

Under these circumstances it is not possible to develop an alternative to the isolation of viruses in hens' eggs and have an adequate vaccine supply to meet public health needs.

For studying antiviral intervention, embryonated hens' eggs can serve as an intermediate between studying antiviral activity in tissue and organ culture, and in small animals. Use of eggs of 9-days or 10- days of embryonic development for this would be usual but some experiments might need to be extended up to and including 14-day old embryos.

Which non-animal alternatives did you consider for use in this project?

Egg-based influenza vaccines remain the mainstay for public health intervention against influenza. Under this current situation, there is no non-animal alternative for this type of activity for the production of such viruses to be developed for use in vaccines and we are committed to making such virus isolates for development into vaccines.

Tissue culture and organ culture are the first steps in analysing antiviral activities but eggs can serve as an intermediate between tissue and organ culture and small animal models. Thereby, eggs serve to reduce the use of animals, and will not usually be done at late stages of development (after 14-days of embryonic development).

Why were they not suitable?

There was no alternative available for producing viruses in hens' eggs for egg-based vaccines, and these remain the main type of influenza vaccine used globally.

In ovo assessment of antiviral treatments serves to reduce animal usage and will typically use eggs at earlier stages of development, and this work will build on work done in tissue and organ culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We need to have produced a number of influenza viruses of each influenza A sub-type (H1N1, H3N2) and influenza B lineage (B/Victoria lineage and B/Yamagata lineage) for initiating the development into new vaccine candidates as the human influenza viruses continually evolve, causing yearly epidemics and occasional pandemics, and warranting new influenza vaccines on a very frequent basis. Also needed are isolates of zoonotic and potentially pandemic influenza viruses.

It is important that sufficient viruses are isolated in hens' eggs are available to screen for ones expected to be able to be developed into a suitable influenza vaccine. The isolation rate of viruses from suitable clinical samples is currently in the order of 50% to 60%. Typically up to around 100 human clinical samples are taken each year for attempted virus isolation in eggs.

Most antiviral experiments done in ovo will be initiated on early-stage embryos and very few eggs will progress to 14-days of embryonic development.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In most cases once a virus has been clearly established as an isolate we can use eggs of lower embryonic age for further propagation, and so not subject to the legislation of the Animal (Scientific Procedures) Act 1986. This reduces the number of animals used under the project licence.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We estimate the numbers from our experience over the last decade that can be developed further. Not all selected viruses will prove to have the properties to take them forward for further development and some of those developed further might not be deemed to be suitable for use as a candidate vaccine virus.

In studying antiviral treatments, eggs of the youngest age (e.g. 9-day old to 11-day old) will be used to initiate the experiments. As above, it is not envisaged that many treatments would extend to 14-days of age.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Embryonated hens' eggs are to be used and these will be inoculated with samples containing influenza viruses into the amniotic and allantoic cavities.

Why can’t you use animals that are less sentient?

Success in the initial isolation and the early passage of virus, notably for influenza A(H3N2) viruses, one of the main causes of epidemic influenza, is best performed using older-aged embryos. This is based on our observations, along with those of others, that as the human influenza virus has evolved the ability to isolate viruses in hens’ eggs has become markedly reduced. This decrease in virus isolation in eggs reduced the number of viruses that could be developed into viruses suitable for influenza vaccines, thus potentially compromising influenza vaccine availability. It was discovered that inoculation of embryos of 14 or 15 days of development, followed by incubation for 3 or 4 days, increased the probability of virus recovery and thus provided us with a reasonable number of candidate viruses that have the potential to be developed into vaccine viruses. Very few embryos, if any, would reach a stage of development when the embryo is deemed to be sentient (1 to 2 days prior to hatching).

Following an isolate being successfully propagated in 14-day old embryos it will be subsequently passaged in 10-day old embryos and incubated for 48 to 72 hours, an age not covered by the law.

Antiviral work will be initiated in 9-day old to 11-day old embryos, but the treatment might extend to or beyond 14-days of development.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Minimisation of any suffering will be done by using embryos less well developed whenever possible. For viruses that might cause the death of the embryo, pilot studies will be carried out to determine the mean time to death in younger aged embryos (typically 9 - to 11-days of embryonic development) and in subsequent work the embryos will be chilled to 4o prior to this time.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The production of viruses in hens' eggs for the development of viruses for development into egg-based human influenza vaccines is an activity done by very few laboratories globally. We will discuss with the other laboratories that carry out similar work whether they discover that younger aged embryos can be used for virus isolation without a

significant reduction in the successful isolation of viruses and whether they have improved procedures.

Work on antiviral treatments will be done according to protocols developed to be robust.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The host institution provides regular updates on advances in the 3Rs. We discuss our work with the small number of other laboratories doing similar work globally.

136. Developing non-viral gene therapies

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

delivery technologies, therapy, non-viral gene medicine, precision medicines

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to develop and test novel non-viral gene medicines for rare genetic diseases and other diseases for which our technology could lead to significant clinical benefits., for example muscular dystrophies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Non-viral gene medicines have great promise as treatment for multiple diseases for which there is currently no (effective) treatment. However, most such medicines suffer from an inability to be effectively delivered, via the blood stream, to target organs in order for them to generate a clinical benefit. We are aiming to develop delivery systems for non-viral gene

medicines, which enable efficient delivery to target organs such that a clinical benefit can be obtained. The nature of our technology is such that there is a potential for its application in many disease areas. We aim to develop new non-viral gene medicines for diseases of muscle, heart and brain for which there are no current treatment options.

What outputs do you think you will see at the end of this project?

Primarily, we expect this project to generate lead candidate compounds for translation to the clinic for the treatment of Duchenne muscular dystrophy, a severe genetic neuromuscular disease. We expect to develop new intellectual property on compounds identified as part of this project. In addition, we intend to publish our findings at scientific meetings and in written peer-reviewed scientific journals.

We aim to advance our first programme for Duchenne muscular dystrophy (DMD) to an application for 'first in-man' studies. In addition, we expect the data generated for this programme will guide the development of our technology for several other gene medicines.

We envisage that the longer-term benefits of this application will be to establish a new technology for gene medicines that allows the rapid development of therapies for both genetic and non-genetic diseases

Who or what will benefit from these outputs, and how?

Developing novel treatments for genetic diseases could have a huge impact on the quality of life of patients and their families. Furthermore, the impact on society in general is potentially significant. For instance, the 'burden of illness' for rare diseases (e.g., Huntington's Disease) are typically high, due to life-long needs of the patient (approx.£70k/pa). However, these costs are typically 45% of the true burden of disease (families providing healthcare and/or requiring adaptations to their homes). NHS England annual spend on rare disease is £3.7b, suggesting the additional costs bore by society in general are £1.7b.

We intend to publish our work in the form of patents and publications in scientific journals which will inform researchers of our new technology.

Long-term, we hope to develop methods to make new non-viral gene medicines, that decrease the time and cost required to get a new drug approved for use in humans. These new approaches could establish new working practices to produce cost-effect therapies for conditions with profound unmet clinical needs.

How will you look to maximise the outputs of this work?

This project will inform the development of gene medicines based on the same technology for additional indications. We have strong links to pharmaceutical companies, all developing similar types of drugs to improve the quality of life. These interactions allow a

freedom of academic exchange that benefits all parties and the success of this project will hopefully lead to inward investment from these companies to develop additional drugs for different diseases. Thus, we expect to positively impact on drug development, both nationally and internationally with a program that reduces the time to market and reduces the extent of animal-based experiments.

To emphasise our commitment to public health understanding, we engage with relevant charities in disease areas of interest to ensure that all drug development programs are made in consideration of a ‘patient-first’ approach. The success of this program could have a positive impact on more global aspects to human health. Our data may be valuable to policy makers in channelling funds to support specific types of research, and regulators developing guidance and legislation for new therapeutic modalities.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mouse has been chosen as the most appropriate species for our studies as it is the lowest form of mammal that allows the study of human diseases. This project is concerned with creating new gene medicines to treat diseases with unmet clinical needs, including muscular dystrophies. In this project we will use mouse strains that have similar genetic mutations to humans suffering from specific conditions and therefore also develop similar symptoms..

New medicines will be investigated to treat the primary cause of the disease (e.g., restore missing proteins), in addition gene medicines will be investigated to treat complications that develop because of the disease (e.g., inflammation and fibrosis).

Diseases can develop in both young and older people depending on the particular disease. Therefore, we plan to use mice over a range of ages (both young and older/adult) to mimic the human disease as closely as possible. This will give the most valid information on the suitability of the gene medicine for treating the disease in question.

Typically, what will be done to an animal used in your project?

Genetically altered mice and mice with naturally occurring diseases (such as muscular dystrophy) will be bred. Before any treatment, non-invasive baseline assessments may be performed (e.g., to assess heart function or general mobility) to enable assessment of the impact any treatment. Muscle regeneration might be induced by intramuscular injection and substances may be administered by intravenous injection to aid drug delivery. Non-

viral gene therapy agents to treat the primary condition or associated pathologies will then be administered either by an intravenous or subcutaneous injection. Non-viral gene therapy agents may also be administered by a small pump inserted under the skin or by oral administration. Depending upon the assessment being conducted, the non-viral gene therapy agent may be given to the mouse once or on multiple occasions. Following the final dose of the non-viral gene therapy agent, the mouse may then be assessed again for any physiological improvements (e.g., heart function, mobility, ability to exercise). Depending on the assessment being conducted, the mice will be sacrificed 1 – 4 weeks following the final dose of the non-viral gene therapy agent and appropriate tissues collected for further analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

All experiments are designed to minimise the number of mice used, with the minimum impact on the mice. Mice might experience minor adverse effects from the administration of the test compounds. They are expected to recover quickly and will be monitored for changes in body weight, condition, assessment of the injection site and food and water consumption. Appropriate modifications to housing, access to wet chow and treatment of injection site will be made if necessary. When surgery is carried out, painkillers and post-operative care will be given in addition to the monitoring described above. No serious adverse effects are expected for the non-invasive physiological assessments.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice: Mild: 85%

Mice: Moderate: 15%

What will happen to animals at the end of this project?

Killed

Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objective of the program is to develop a series of novel gene medicines to treat neuromuscular, neurodegenerative, cardiac and metabolic diseases. In order to support the development of medicines intended for human use, from therapeutic concept to human clinical testing, animal testing is a necessary requirement to assess whole-body responses to novel medicines which cannot be assessed in non-human or non-animal models.

Which non-animal alternatives did you consider for use in this project?

First, our programs will be informed by the current practices and what has already been published. In most aspects of our programmes, initial testing and assessment of novel compounds will be carried out using computer programs or cells cultured in an incubator. This allows predictions to be made as to the suitability of each synthetic gene medicine before it enters experiments in animals. We have developed a number of tests, using cells grown in an incubator that provide key data about potential toxicity and efficacy of our candidate synthetic gene medicines; all these tests lead to higher quality data being produced and a reduction of the number of animal required. These hard stop:go outputs derived from a computer program and/or cells in culture support our ‘fail fast’ philosophy and the increased likelihood of clinical translation of the respective programs.

Why were they not suitable?

The target organs for the respective gene medicine/indication combinations are complex tissues that responds to environmental influences in a number of ways and, as such, cells in culture and whole organ studies are not suited to the full assessments of the potential therapeutic benefits; nor, due to the complex interaction that each tissue has with its environment, would it be possible to fully assess the benefits of our gene medicines

In addition, cells of skeletal muscle, heart and brain typically do no undergo cell division in adults. In particular contractile cells of the heart (cardiomyocytes) do not divide and there are no cell lines that are representative of these cells. It is therefore necessary to use animals for such studies evaluating cardiomyocyte function within the intact heart, as there are no non-animal alternatives.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal experiments will require pilot studies to determine whether our experimental approach is worthy of further study. Such studies also can inform on the numbers of animals required based on the early observations of the effect of our new gene medicine.

In addition, animal number requirements will be informed by published data from studies by others using the same animal models, where possible. Based on our previous work, for each new pipeline program in which pilot studies, safety studies and efficacy studies are conducted, based on the types of disease under investigation 200 - 400 mice are typically used; this number enables the collection of robust data sets that supports the continuance of the pipeline towards first-in human studies.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To reduce the number of animals used in the project, we have developed a mathematical process that includes the experimental design and methods of analysis of the results. This process will be ongoing in collaboration with trained statisticians and reviewed within our team. Such training programs are reviewed annually with new staff undertaking an induction statistical program to cover how to produce high quality data with best outcomes commensurate with the 3R's. With appropriate statistical analysis being performed, both the qualitative and quantitative measures collected will be maximised. In addition to our review process, our participation in numerous 'networks of excellence', together with the literature will inform on optimal experimental design that will positively impact on the numbers of animals required, yet ensuring the delivery of clinically meaningful benefits.

We build out and internally disseminate a stepwise visual representation of our planned animal experimentation. This promotes greater critical assessments of our workflows and provides feedback and advice from colleagues with considerable animal experience. This iterative approach establishes best practice to define the relevant preclinical (and therefore, clinical) outcome measures and accompanying appropriate analyses.

Our practices, thus comply with the NC3Rs PREPARE guidelines, that further disseminate best practice to the wider community as a whole. With memberships of numerous 'Networks of Excellence' (groupings of experts that share best practice) this sharing of information leads to standardised operating procedures permitting greater comparisons between studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the outset of establishing novel gene medicines for a new disease indication, prior to establishing a new pre-clinical mouse model, we will seek opportunities to obtain tissues from others that have archival tissue material. This helps establish a set of parameters to best identify the types of pathological changes that would be of interest when in vivo studies are conducted. Our experience is that this approach reduces the numbers of animals required per program.

We will aim to limit the numbers of mice produced for our program by collaboration with external service providers; Where possible, animals will be obtained from centralised breeding facilities following advance planning taking into account preparation time for

planned experimentation. In addition, where possible both male and female animals will be used for our studies which will separately contribute to the reduction of surplus animals.

For experiments where breeding of animals needs to be done in-house because the animal model cannot be obtained from centralized breeding facilities, clear estimates of animal numbers required for experiments will be produced ahead of time to determine the appropriate number of breeders to use to minimise surplus animals.

The incorporation of computer-based and cell culture-based screens prior to any animal study will minimise the number of compounds tested in animals and therefore, the number of animals used.

We have developed appropriate non-invasive outcome parameters that allow repeated measurements on an animal which promotes a greater level of data being generated per individual study AND individual animal. For post-mortem tissue analyses, we have established protocols to assess functional, histological, and molecular parameters from the same tissue samples; this multiple sampling data generation from the same tissue leads to the reduction of animals required to obtain the data to progress the program into clinical trials.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pre-clinical mouse models available for our studies are based on the same genetic defects that result in the specific human condition under evaluation, for example muscular dystrophy. Together, there has been considerable data, often collect over tens of years, that has convinced medicine regulators globally of the relevance that such models have, with particular reference to the type of gene medicines we are analysing. For example, models of dystrophic mice are accepted as ideal systems in which to conduct early evaluations of novel therapeutic agents. Delivery of non-viral gene therapy agents by injection or through food will allow us to study the effect of these new medicines on the primary disease and pathologies that are caused as a complication of the condition.

All our experiments are designed to cause the minimum amount of pain, distress, or lasting harm; this is done by adopting standard operating procedures and training by trained and competent staff.

Why can’t you use animals that are less sentient?

The mouse is the lowest sentient mammal used in this type of study to inform on the human condition. There is significant overlap between mice and men in molecular and cellular programmes during development and/or regeneration. Other experimental models, e.g., fruit flies, nematode worms and fish, do not share these characteristics and hence extensive studies have been performed in rodents as they are appropriate models to assess the suitability of specific therapeutic regimens. In addition, we have developed protocols that allow us to interfere with gene function in rodents which aid the study of tissue functions or metabolism in our relevant models, including muscular dystrophy and metabolic disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Suffering will be minimized by the use score sheet to assess the severity of experimental interventions and the use of appropriate humane endpoints. Animal husbandry will be modified where appropriate (e.g., floor feeding). Adopting non-invasive imaging and functional assessments will permit longitudinal studies and lead to a refinement in humane pre-clinical endpoints and improve the quality of the data. Animals will be continuously assessed and scored against recognised welfare parameters to ensure pain, suffering, distress, or lasting harm to the animals does not occur.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Throughout our workstreams we will adopt a 4 step approach to Animal Research

9. Appraisal of best practice as described by the Laboratory Animal Science Association https://www.lasa.co.uk/current_publications/

10. Use PREPARE guidelines in concert with the animal facilities to plan any animal research programs to minimise use and/or levels of harm. https://norecopa.no/PREPARE

11. Highlight and promote improvements that have impacted on the 3R’s; not being restricted to prepared manuscripts.

12. Comply with the ARRIVE guidelines to ensure best practice and report outcomes are communicated effectively to engender and comply with best practice. https://arriveguidelines.org/arrive- guidelines

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Best practice dictates that one is guided by the literature and from our own experiences. Keeping abreast of the developments in science (via appraisal of the literature, scientific Networks of Excellence, scientific meetings or press releases and/or news letters from key

opinion leader (e.g., NC3Rs) will ensure constant reflection and, where necessary, adoption of modified practices that will conform with any ‘new’ best practice.

137. Molecular mechanisms of t cell mediated immune responses

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Immune response, Egr, Tbet, autoimmunity, Tumor microenvironment

Animal types	Life stages
Mice	adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Based on our recent findings of the function of early growth response gene 2 (Egr2) in T cells and the establishment of therapeutic vaccines, this project has two aims:

1) Define Egr2 controlled molecules in self-reactive T cells for the development of diagnostic biomarkers used for autoimmune diseases,

2) Develop a therapeutic vaccine for treating solid tumours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Both autoimmune diseases and cancer result at least partly from the dysfunction of T cells. The regulatory mechanisms in T cells hold the key to not only effective immune responses against pathogens and cancer but also the ability to tolerate self-tissues. The disorders of such mechanisms will lead to autoimmune diseases as well as impaired anti-cancer responses. We have discovered two molecules in T cells, early growth response gene (Egr) 2 and 3, which have a reciprocal function in promoting the anti-pathogen function of T cells while controlling their inflammation. This function is essential for both optimal immune responses against pathogens and cancers, and also preventing the development of autoimmune diseases. Recently, we discovered that the anti-inflammation function of Egr2 in T cells is due to the inhibition of another molecule, transcription factor T-box expressed in T cells (Tbet). Tbet in T cells causes inflammation, leading to a potentially dangerous autoimmune reaction. Therefore, this mechanism for controlling Tbet will be applied for the diagnosis and novel therapy of autoimmune diseases. With the understanding of factors regulating the function of T cells, we have also developed a therapeutic vaccine that can up-regulate the anti-cancer responses of T cells.

Based on our findings and newly established Egr2-Tbet transgenic mouse models, this project aims to uncover the molecular mechanisms in preventing autoimmune diseases and promoting anti-cancer responses. The discoveries will be applied for the development of diagnosis and novel therapeutics for autoimmune diseases and cancer.

What outputs do you think you will see at the end of this project?

It has been established that dysfunction of T cells can lead to both autoimmune diseases and cancer. The poor understanding of the reasons leading to this dysfunction limits the development of new diagnostic methods and treatment. One of the major outcomes of this study is to apply Egr2-regulated molecules in activated T cells as early markers for inflammation of autoimmune diseases, so it is possible to allow effective management of these patient’s illnesses. At the end of the project, we will not only publish our findings in leading journals, but crucially, provide scientific base for applying these markers in lupus and arthritis clinics.

The other major outcome is to develop a novel immune therapy for individual treatment of cancer. Microsome vaccine developed in this project was made by isolated membranes from surgical removed tumour tissues. We will use our models to refine the productive methods involved and try to combine this with other immune treatments to maximize efficacy. At the endpoint, we will have enough results to apply grants for translating our findings in mouse models to develop therapeutic vaccines for the treatment of solid cancers such as lung and pancreas cancer.

Who or what will benefit from these outputs, and how?

In short term, our findings (with in the PPL period) will benefit researchers in both basic and clinical groups to understand the mechanisms of auto-reactive T cells in disease

models and the new form of vaccine delivery systems for cancer vaccines. These benefits will be realised soon after we have results from phenotypes and clear assays of the T cells from our models and from efficacy analysis of vaccine treated tumour bearing mice. As this study is closely collaborated with our clinical investigation in T cells from the patients of autoimmune diseases and cancer, the findings after completing this project will be ready to translate into clinical applications.

The knowledge transfer will be realised after second year of the project, some of our findings will start to be translated as clinical tools for autoimmune clinics and at the end of the project hopefully we will apply our findings into translational research to develop diagnostic methods and novel vaccine for diagnosis and treatment for relevant human diseases.

How will you look to maximise the outputs of this work?

We are an established research group in T cell biology and have collaborators across the world. This project is the major piece of active research within our group. The discoveries will be published in high impact journals and international conferences. We will communicate and report all of our research approaches within our field and offer newly developed tools and models to other groups. Importantly, we have established close collaborations with three major clinical research institutes in the UK, Germany and China in cancer immunology and arthritis studies. The support of this global network will ensure the highest possible impact of our findings.

Species and numbers of animals expected to be used

- Mice: 3800

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Systemic analysis of immune function can only be done under living conditions and it is impossible to use humans as experimental models. We have chosen to use mice since the parallels between the mouse and the human immune systems are well understood and mouse models of the diseases are well established. This means that reagents are readily available and obviates the need to establish novel models which greatly reduces animal use.

Adult mice will be used to assess the immune function when immune systems are fully developed.

Typically, what will be done to an animal used in your project?

The following procedures will be carried out some examples what will be done as following:

- collect lymphoid organs from culled mice (at the end of most experiment and procedures).

- Breeding transgenic mice (for breeding and maintenance of genetically altered protocols, duration of less one year);

- Injection of immune modulators such OVA/SEA or microsome ip.(in the protocol of In vivo immune responses, with repeated 2 times per animal, duration of less 10 weeks);

-Adoptive transfer of isolated T cells from one type of mice to other, iv,(in the protocol of adoptive transfer of immune responses, once per animal, duration of less 12 weeks);

- Irradiation of live mice for bone marrow transfer (in the protocol of tumour prevention and treatment, once per animal, less 12 weeks);

-Injection of tumour cells subcutaneously (in the protocol of tumour prevention and treatment, once per animal, less then 8 weeks, but tumour growth will be limited less than diameter of 10 mm);

- infection with recombinant vaccinia virus i.p.(in the protocol of viral prevention, less 2 times per animal, less than 12 weeks).

What are the expected impacts and/or adverse effects for the animals during your project?

The impacts could be as following:

- Local pain from injection for a few minutes, but less 48 hr (in most procedures involved).

- Weight loss after viral infection for 3 to 7 days (eg, in protocol of viral prevention), but the weight loss will be limited no more than 20%)

- Tumour grow for 7-14 days (in the protocol of tumor prevention and treatment), but tumor will be limited

< 10mm diameter.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are no more than moderate which only occur in less than 20% of experimental mice in all protocols based on previous my PPL experiences with similar procedures as below:

- for protocol of "Breeding and maintenance of genetically altered with moderate protocol": 1500 mice; Moderate.

- for the protocol of "In vivo immune responses": 700 adult mice; Moderate.

- for the protocol of "Adoptive transfer of immune responses": 600 adult mice; Moderate.

- for the protocol of "Tumour prevention and treatment": 1000 adult mice; Moderate.

- for the protocol of "Viral prevention": 1000 adult mice; Moderate.

-for the protocol of "Breeding and maintenance of genetically altered with mild protocol", 1800 mice; Mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To understand the mechanism of key molecules in T cells, a systemic analysis of the immune system under living conditions is not possible with humans in vitro. Therefore, animal models are the only solution.

We have chosen to use mice since the parallels between the mouse and the human immune systems are well understood and mouse models of the diseases are well established. This means that reagents are readily available and obviates the need to establish novel models which greatly reduces animal use.

We will carry out, if possible, pre-experiments on cell lines to optimize the procedures and ensure the success of the animal experiments.

Which non-animal alternatives did you consider for use in this project?

We will assess mechanisms of molecular interaction using cells in vitro (after adding or removing Egr2) in a laboratory without using alive animals. Thus, the number of animals used in this project is limited only for the specific analysis of systemic functions of T cells in disease conditions.

Why were they not suitable?

Immune function is systemic and only established in living animals. Therefore, it is impossible to use a cellular system to complete the project. The function of Egr2 and 3, and Tbet in T cells can only be revealed during immune reaction in disease models in living animals. The gain (with gene high level) and loss (genes defected) approaches are more effective methods to understand the function of molecules such as Egr2 and Tbet. Therefore, we have genetically modified the Egr2, Egr3 and Tbet in T cells to control their presence or absence during immune responses.

We cannot carry out any of these experiments in humans.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Due to the factors that the experimental models used are very well established and used worldwide, we have used all the models in our previous three PPL projects and the minimum requirement for measuring T cell function in systemic immune responses are well defined in our animal models.

Therefore, in this project we can accurately estimate the number of animals needed. For all phenotype analysis, differences between groups of mice will be assessed by effective statistical analysis, such as power calculations will be performed to determine the minimal number of animals needed to obtain statistically significant data dependent upon the level of effect for the parameters being assessed. Thus, if the size of the effect is equal to a standardised difference of the mean of between 1-2, each experimental group will contain between 4 and 16 mice with age- and sex-matched, which will allow a significance level of 0.05 with 80% power to be achieved. Scientifically, each experiment has to be repeated at least three times. However, one of the aims of this project is to define the molecular signatures in T cells after immune reactive to experimental diseases. In this part, we have established refined molecular approach to detect molecular differences in a minimum of 50000 cells. Therefore, we will use three to five mice in one experiment and isolate cells (mainly lymphocytes) from each mouse, then compare the difference between mice in same group to reach scientific conclusion. In tumour vaccine experiments, a minimum of 5 mice has to be used and at least repeating three time to reach scientific conclusion. With refined technology in cell lines and well experienced protocols for tumour vaccine, we can estimate the number of animals used.

A total of around 3800 mice will be needed for the PPL applied. As average homozygous production rate in transgenic lines is about 75%, about 2800 transgenic mice needed to reach enough 2200 homozygous mice, so total 3300 (with 500 breeding mice) for GA breeding protocols (including the moderate breeding and mild breeding), which will be used for the experiment: 700 for protocol of in vivo immune responses; 600 for protocol of adoptive transfer of immune responses; 1000 for protocol of tumour prevention and treatment; and 1000 for protocol of viral prevention.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will plan the experiments that can allow us to monitor as many biological parameters as possible in T cells, such as identifying DNA bindings of Egr2 in T cells from virus infected mice. Thus, we could use one designed experiment to answer multiple questions.

We will plan experiments, including breeding strategies, in advance so that breeding will be limited as necessary. Where possible, randomised block and factorial designs will be introduced into experimental strategy in order to minimise the number of animals used.

We will focus on three elements in our design to reduce number: 1) do pre-experiments in cell lines for in vitro methods such as phenotyping, RNA sequence and chromatin remodelling to optimise protocols before starting in vivo experiments; 2) combine different experiments in one animal experiment such as identification of prototypes and analyse chromatin remodelling of effector T cells are combined in same viral infection experiment; and 3) extensive literature searches, inform and communicate with related labs/groups to avoid necessary replication of the experiments that have been reported. We will also maximise use of tissue, and genetically altered animal lines to maximum the efficiency to get the results.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have good experiences in animal experiments and the models used in this project which allow us to design breeding program together with experiments. We are closely collaborating with groups sharing similar interests in T cell biology and extensively analyse reported database to avoid repeated experiments. We have published modelling of T cell function regulated by Egr2 that allows us to focus on key questions investigated by well-designed experiments. These will reduce significantly the number of animals used in this project.

All the experiments will be designed with not only the concurrent and relevant to make the outputs addressing the proposed question directly, but also with accurate repetition and controls based on randomisation, power calculation and biological relevant. At same time, we will avoid bias, repetitive to other reported experiments and maximising data acquisition in each experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are going use mouse model. Mice are considered to be a mammalian organism similar enough to humans to provide invaluable information from pre-clinical testing of potential therapeutic approaches before progressing to human clinical trial.

As the aim of project is to study the role of molecules Egr2 and Tbet for the function of immune system, we are going to use Egr2 and Tbet related transgenic mouse models for this project, so that the experiment system is sensitive and clear for the purpose, and to maximum the efficiency of experiment result output.

The methods used in this project, eg, we will infect mice with modified experimental virus which can activate immune system but not cause severe symptoms; injection of well-established tumour cells under skin which will result in local tumour and tumour induced immunity, but will not cause organ damage; transfer of T cells from one type mice to other to define specific function of genetic modified T cells in normal mice, and bone marrow transfer to assess the function of newly developed effector T cells from donor mice in recipient mice.

Why can’t you use animals that are less sentient?

Mice are the lowest vertebrate animals in which immunological studies are possible to conduct.

Immune system is essential for keeping wellbeing of normal life of human being. Closely related and less sentient animal is mouse. Therefore, majority of immune science are now investigated in mouse models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In this project, we only use well-established disease models that have well defined clinical symptoms and will control the level of clinical sign to minimum severity.

In addition, we will follow the best practice with all the procedures as much as possible to reduce welfare costs, for examples:

-To optimize breeding program of EGR KO mice in order to avoid welfare problems in offspring (protocol of breeding and maintenance of genetically altered with moderate): Previously, it was found that the failure of some mice to thrive in the first 21 days of life with slow growth rate and less activity than normal is due to “tooth development problem" with Egr3 deficiency. Moreover, the double KO female mice usually had less care for their offspring. To avoid these problems, a special breeding strategy has been considered by using Egr2homo/Egr3homo (male) with Egr2homo/Egr3 heter (female) for the breeding in order to improve the health of offspring and we will regularly check the new-burns to eliminate the problem at the earliest time point (e.g., by giving some mashed food around 20 days; keeping the KO babies with the breeding females for one month instead of 21 days).

- For microsome vaccine test in tumour models (protocol of tumour prevention and treatment): only subcutaneously route for the tumor injection will be used as it is less harmful to the mice and easy monitoring the tumour growing comparing any other injection methods.

- For viral infection study (protocol of viral prevention): when vaccinia viral system is used with intranasal (i/n) route the mice showed clear clinic symptoms but more stressed with a lower food intake, less active and loss more body weight compared with intraperitoneal (i/p) route. However, for the purpose of investigation of T cell responses in this PPL, the study can be achieved using i/p. instead of i/n., which would have less harmful to the mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The disease models and bone marrow transfer are well established in countless publications from other and us. In which the unified practice guidance is adapted worldwide. We will stick on the the most practice guidance which have been reported in our publications and accessed many times by local ethic committee and Home office.

We will follow best practice based on published data, below as some examples

- For Reconstitution of bone marrow (protocol of adoptive transfer of immune responses): A fractionated dose for whole body irradiation using “2X of 5.5 Gy with 3 hours apart" will be less harmful for the animal. Furthermore, we have recently been able to refine the irradiation process by optimising the orientation of mice to the irradiation source which is less restraint to the mice.

- For viral infection and tumour in vivo study (protocols of tumour prevention and treatment and viral prevention): It is not possible to study the mechanisms of Egr2 and 3 in the development of T cell function without using animal model. We have established best possible experimental models in both viral infection and tumour to achieve the induction of T cell immune responses with minimum discomfort. In virus infection, we have refined protocols that results full immune responses in infected mice within 20% of body weight

loss. In tumour models, the local growth with limitation of 1cm size will induce detectable anti-tumour immune responses with limited stress to the animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are constantly communicating with top level scientists in our field to renew our technologies to avoid unnecessary mouse experiments. We carry out pre-experimental tests in cell lines, and molecular network modelling to refine the mouse experiments at maximum scientific outcome and minimize the use of animals. We have well implemented these in our recent research. With our regular meetings which a 3Rs agenda is regularly discussed and updated, we share 3Rs news including technical updates, relevant publications and meetings or conferences.

Home Office

138. Understanding immune cell development and its functional consequences

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immune cell development, Immune cell function, T cells

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project seeks to better understand the mechanisms that underpin the development of immune cells; that are critical effectors in all immune responses. Importantly, it will focus on how development of immune cells impacts on their subsequent function.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding how the immune system functions to combat infections and malignancy, while at the same time being tolerant to our own tissues and harmless surroundings, has never been more important. Critical insight into these complex functions comes from a thorough understanding of how these cells are generated. Thus, this project focuses on the mechanisms by which immune cells develop and how this influences subsequent immune cell function.

What outputs do you think you will see at the end of this project?

The studies detailed in this proposal will significantly advance the understanding of how immune cells develop, particularly T cells that are generated in the thymus. These new insights will be communicated at National and International conferences (e.g. as poster or oral presentations). We will also publish key findings that advance the field in journals that have wide-ranging reach and impact, with a particular emphasis on open-access publication and data sharing.

Who or what will benefit from these outputs, and how?

A key focus of this work is the development of immune cells (known as "unconventional" lymphocytes) that are increasingly considered to connect the broadly reactive, rapidly acting innate immune system with the delayed responses of the pathogen-specific adaptive immune system. A better understanding of the development of unconventional lymphocytes will provide critical insight into how they function. In turn, this will better inform manipulation of unconventional lymphocyte responses in disease, and will better advise how these cells can be utilised in therapeutic strategies, for example to augment anti-tumour immune responses.

Thus, in the short-term benefits of these outputs would be to other researchers in the field of lymphocyte biology (e.g. developmental immunologists, T cell and B cell biologists). In the mid-term, these outputs may advise modifications to the development and use of unconventional lymphocytes in immuno- therapeutic strategies, particularly in the cancer setting. And finally, long-term benefits may be to patients who benefit from therapies that utilise immune cells to ameliorate chronic and potentially fatal diseases.

How will you look to maximise the outputs of this work?

We have a strong track record of maximising the impact and reach of publication outputs through international collaborations. We have also made regular contributions to the field in the form of review, opinion, and perspective articles, and talks at National and International conferences, in which unpublished work, concepts, ideas and plans are discussed with the scientific community.

Species and numbers of animals expected to be used

- Mice: 9000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Genetically modified animals will be used either because no other suitable tools exist, or because a modification that cannot be achieved by other means is required.

Genetically modified mice are ideal for these investigations as they have been the model of choice for studies on the development and function of the immune system for decades. Thus, data generated form these investigations can be analysed and understood in the context of the wealth of previously obtained information related to the subject.

Animals will be culled by schedule 1 methods to obtain tissues from mice at embryonic stage day-11 (E11) through to adult mice (<15-months). It is important for this project on lymphocyte development to use embryonic and neonatal stages as development of the immune largely occurs between the ages of E11 to 3-weeks of age.

Typically, what will be done to an animal used in your project?

Typically, an animal used in this project will be bred and maintained and then killed by schedule 1 methods (or under terminal anaesthesia by exsanguination then schedule 1 method, or fixation perfusion as described above), at any age from embryonic stages to adult mice (<15-months).

Depending on the genetic modification an animal may have a tissue biopsy taken to determine genetic status by one of the following methods: ear punch, blood sampling, hair sampling (rarely, due to technical problems during analysis, a second sample may be taken using the least invasive method).

For strains with inducible genetic alterations, animals will be injected or treated with appropriate compounds in order to activate the specific genetic alteration. Mostly mild effects are expected from this dosing, but some animals may show some transient moderate effects. In all cases of administration of substances, we will follow LASA and Refining procedures for the administration of substances (Morton et al. 2000) guidelines. Animals will be monitored daily during the dosing days for general health and any animal showing any unexpected distressful response (no activity, unresponsive, hunched, loss body weight to 20%) will be culled by a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

The phenotypes of the mouse strains used in this project are anticipated to have no harmful effects.

The substances that we will use in this project to induce genetic alterations in mice with inducible genetic modifications have been used previously in other laboratories. They are widely used substances for which the toxicity and side effects are well known, e.g. doxorubicin and tamoxifen. These genetic alteration-inducing protocols are highly unlikely to cause adverse events of more than mild, short and temporary harm.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

Expected severity for breeding and maintaining GA animals is mild; >99% sub-threshold. Expected severity for induction of inducible genetic alterations in mice is mild (100%).

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

These studies aim to define the molecular basis for the dynamic interactions of different cell types within the distinct spatial anatomy of organs where immune cells develop, such as the thymus, bone marrow, and gut. Such complex, dynamic interactions cannot be adequately represented by sole use of cell lines, models in vitro, or mathematical models. However, as we learn more about the key interactions between cell types, e.g. within the thymus or gut, we shall maximise our efforts to use organ cultures for dissecting key pathways, as we have published. Nonetheless, we require live animal models to accept or to refute hypotheses concerning the molecular basis of lymphocyte development, and the pathophysiologic importance thereof for human beings. As such knowledge emerges, our collaborations with various clinical research teams will promptly investigate the parallels using human cells and clinical material.

Which non-animal alternatives did you consider for use in this project?

We have considered and will continue to consider the use of non-animal alternatives. These include in vitro organ culture, and exploring the use of "organ-on-a-chip technologies. We also utilise various types of thymic organ culture, using thymic tissue ex vivo, for the vast majority of our experimental investigations, to avoid doing these in intact animals in vivo. We are also actively looking at ways to reduce the use of animals in research by developing better in vitro models.

Why were they not suitable?

We use thymic culture techniques in the lab whenever feasible. However, none of these, as yet, fully reproduce the dynamic interplay between different cell types within the distinct

spatial anatomy of organs where immune cells develop. Thus, we still require tissues ex vivo to set-up and seed many of our culture systems.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

During similar work under the animal licence of our collaborator we calculated that we bred and used 150 animals in an average month. For any one month, this would represent 30 animals (approximately four litters) from five mouse strains. This would supply the necessary tissues (largely thymus) for our program of experiments ex vivo. In addition, we are using the NC3Rs EDA tool to assess individual experimental design. This ensures that the correct number of animals are used per experiment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our Institute statistician was consulted during the experimental design phase of the project. The NC3R website also contains many useful guides for experimental design, e.g. NC3R's Experimental Design Assistant. Moreover, most of work will be directly linked to appropriate breeding colony management, and our team has sound expertise on GAA breeding, according with international guidelines (Jax Labs US-https://www.research.uci.edu/forms/docs/iacuc/JAX-breeding-strategies.pdf).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We are continually looking to refine and improve our methodologies for running organ cultures in vitro. For example, better utilisation of stromal cell lines that support lymphocyte development would reduce the number of mice required to supply primary stromal cells ex vivo. We are also exploring new technologies, for example organ-on-a-chip approaches.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used in this project to provide tissues (primarily thymus) for experiments and culture systems in vitro. The genetically-modified strains of mice used to provide these tissues are either "knockout" mice, or 'Knock-in" mice with a deficiency or increase in a particular gene product, respectively. These either affect the presence or absence of particular immune cells (e.g. Rag-1 KO mice that lack all lymphocytes), or affect a particular signalling pathway (e.g. constitutively active signalling "knock-in" mice). Generally, these mice do not have phenotypes that impact negatively on the health or well-being of the animals. Mice generated and maintained to supply these tissues with be killed using schedule 1 methods. Thus, pain, suffering, distress, or lasting harm is not expected in our protocols and will be kept to an absolute minimum (e.g. tissue sampling for genotyping).

Why can't you use animals that are less sentient?

We will use mice in this project as the basic physiology of immune cell development is sufficiently compatible with human, and already well-characterised (hence, a better context for new findings). In addition, the mouse model allows for use of genetically altered animals, of which there are now thousands. Note that humans and mice have a comparatively similar immune system that is also observed in most jawed vertebrates (although fish are somewhat different too). More primitive animals (e.g. nematodes, drosophila) do not have comparable immune systems so would not be an appropriate model to study lymphocyte development.

As the primary focus of this project is the development of immune cells, tissues are used primarily from young animals (embryos, neonates, and mice between 1-6-weeks of age), as choosing these ages better captures the developmental process. Occasionally we will use older adult mice (as a comparator) up to 12-months of age.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The protocols for breeding and maintenance of the genetically-modified animals in this project are mild (as are the strain phenotypes), with <1% expected mild or short-term moderate adverse events.

Nonetheless, we will stay up-to-date with the latest recommended protocols, e.g. when performing tissue biopsies to determine genetic status by one of the following methods: ear punch, blood sampling, hair sampling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Regular reference will be made to the Home Office homepage and documents, NC3R homepage and documents, and ARRIVE or PREPARE guidelines. This will ensure full transparency in reporting all the in vivo experimental data. In all cases of administration of substances, we will follow LASA and Refining procedures for the administration of substances (Morton et al. 2000) guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The latest advances in the 3Rs will be obtained through information/newsletters from the Home office and NC3Rs, attendance at relevant academic conferences, and regular literature searches. Identified new methods/protocols will be discussed with our Biological Sciences Unit staff, NVS and Home Office Inspector.

139. Mechanisms of cardiac arrhythmogenesis – insights from murine model

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

heart, arrhythmia, mitochondria, ion channel, electrophysiology

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall purpose of our project is to determine the link between the genetic causes of inherited arrhythmic syndromes and more general phenotypes associated with acquired arrhythmias. In doing so, we aim to advance understanding of arrhythmogenic mechanisms in order to aid future therapeutic and clinical intervention.

Our project is defined by the following key elements:

1. The mechanisms underlying arrhythmias caused by genetic perturbations

2. The possibility for drugs restoring the function of ion channels and metabolic regulators affected by such genetic perturbations to rescue atrial and ventricular arrhythmias.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding mechanisms for arrhythmia particularly in ageing remains a main stay in the diagnosis, treatment and prevention of cardiac arrhythmia. In addressing this global challenge, the use of animal models with targeted disruption of specific physiological pathways provide valuable insights into the elucidation of arrhythmogenic mechanisms. Concerns and lack of fundamental mechanistic understanding with regards to ageing and sodium channel pathologies has led to limitation in how new drugs are develop and an aged arrhythmic patient is managed in clinics.

Our work is fundamental to the understanding of arrhythmia and its propagation, a question of particular importance to electrophysiologists, but also importance to the study of biological pattern generation associated with drug administration and ageing. It will link the underlying pharmacology to cell membrane and intra-cellular currents, bridging research between drug-induced disturbances and physiological effects. It will allow this novel paradigm to be scrutinized as a platform for analysis of electrophysiological abnormalities in a range of pharmacological agents. The work will be of relevance to pharmaceutical researchers and cell biologists, who will gain insight into the role of ageing and selected drugs in cardiac function and correlation with electrical and cellular abnormalities.

What outputs do you think you will see at the end of this project?

Beyond the inevitable short-term output of an enhancement in our foundational scientific understanding of arrhythmogenic mechanisms by the elucidation of candidate molecules and associated signalling pathways, our work will have even more direct clinical implications. Notably, by addressing our objectives outlined previously, our expected long-term outputs are the identification and vetting of: (1) biomarkers for improved diagnoses and prognoses7; and (2) pharmacological targets for the development of efficacious novel drugs8. We aim to publish our data in high-impact academic journals,
national and international conferences, and, by extension, disseminate them to the wider, global community of cardiac electrophysiologists – a feat we believe highly likely given our publication of over 60 papers in the last years and our presentation at more than 80 events.

Who or what will benefit from these outputs, and how?

Given that, every year, there are 50-100 Sudden Cardiac Deaths (SCDs) per 100,000 members of the population in Europe and the USA, the translation of our findings based on the laboratory Genetically- Modified (GM) murine model into diagnostic tests and pharmacological or other therapies at clinicians' disposal will undoubtedly be perceived as worthwhile by the patients who benefit from them.

Additionally the pharmaceutical industry or biotech companies may also benefit from this work given that we may potentially identify new 'drug-able' targets. A corollary of patient benefit is, of course, the diminished economic and health burden of arrhythmias on the NHS.

How will you look to maximise the outputs of this work?

Our experience from previous projects, as well as through our collaborators, who have held Project Licences in the past, ensures we will minimise pursuit of unproductive lines of enquiry, such as a drug soon identified as producing unacceptable side-effects. Likewise, this will maximise pursuit of novel avenues on signalling molecules and drug targets that exhibit early promise. Importantly, we aim to greatly facilitate the clinical limb of our translational research program through fostering a link with NHS hospitals, using the results of laboratory experiments involving our transgenic mouse model to focus on the electrophysiological assessment potentially in patients.

Likelihood of achieving benefits with these models and methods

All protocols and techniques necessary for the generation of monogenic perturbations in mouse lines such as those described above have been well established by scientific consensus in the electrophysiology community and refined through our own experience in using similar Genetically- Modified (GM) murine models for 15 years under the previous Project Licences. Given the critical role that ion channels permitting currents play in the waveform of the cardiac action potential, it is highly likely that any metabolic regulator directly or indirectly modulating channel gating properties will precipitate arrhythmogenesis when either absent or overexpressed in our GM mice lines. Furthermore, owing to the function of metabolic regulators such as PGC1 as transcriptional coactivators of genes upregulated by nuclear receptors, coupled with the observed and highly substantiated contribution of an aberrant cardiac transcriptional profile to arrhythmias, our analysis of age-related homeostatic disturbances is also very likely to identify mediators of the link between metabolic dysregulation (as in obesity and diabetes mellitus) and arrhythmias.

Of the completed series of experiments working with previous collaborators, many have provided a basis on which to conduct predictive testing. Importantly, the identification of high-risk patients permits rapid instigation of protective measures, such as the installation of an implantable cardiac defibrillator (ICD), to reduce the incidence of sudden cardiac deaths (SCDs) that would otherwise arise from arrhythmic events. In fact, such diagnostic utility is evident in the implantation of more than 15,000 ICDs globally. Moreover, the use of our GM mice to understand the basis of arrhythmogenic risk in monogenic disorders has

already facilitated, and will continue to permit, characterisation of molecular targets for therapeutic, and especially pharmacological, intervention. Our GM mouse model conveniently provides a means for testing not only the efficacy, but also the safety, of any pharmacological agent. We thus consider it highly likely that, with such links, our generation of knowledge on the biochemical structure, biophysics and molecular interactions of putative targets will drive future development of antiarrhythmic drugs specific to patients with metabolic disease, with the long-term aim of reaching phase I clinical trials.

Species and numbers of animals expected to be used

- Mice: 240

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

These animals have been validated to provide the most translational results that will closely resemble conditions affecting humans. It is not possible to undertake such experimental interrogations in humans.

We are aiming to study the effects of certain gene mutations in the ageing process. We will use mice up to the age of 24 months old since mice ranging from 18-24 months of age correlate with humans ranging from 56-69 years of age. This age range meets the definition of “old,” which is the presence of senescent changes in almost all biomarkers in all animals. We will not use mice older than 24 months as mice are then considered “very old” and survivorship drops off markedly.

Typically, what will be done to an animal used in your project?

Breeding, ear clipping for identification (ear notching), maintenance up to 24 months of age, drugs administration, ECG recording under anaesthesia and surgery for telemetry under anaesthesia.

Humane killing with a Schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

Pain during injections can occur. Otherwise, any other adverse effects may be related to the normal ageing process. For this reason, animals will be monitored regularly. Moderate to long term administration of substances/drugs can cause repeated incidences of mild pain and therefore stress to the animal. Additionally, this is coupled with the stress of repeated handling during the injection or oral gavage procedure. Insertion of needle ECG,

whilst done under anaesthesia, may have some minimal lasting mild soreness at needle entry site. Animals that undergo surgery will have incision wounds that will take time to heal and this can cause low grade inflammation and pain over the healing process.

There may be some initial discomfort due to post-surgical anaesthesia or due to the device implanted within the body cavity.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate – We predict that approximately 50% of animals to be used in this license will be subjected to Protocol 3 and thus would be subjected to moderate severity. This is estimated at approximately 240 mice.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The heart is a complex organ with several processes occurring simultaneously under physiological and pathophysiological conditions (e.g. organelle and cellular interactions , dynamic metabolic changes, hormonal alterations and more). The ageing body, especially when coupled with other conditions such as obesity and diabetes add to the complexity of the studied processes. Hence, compared to cellular or tissue models, an animal model is better suited in studying physiological processes in the heart.

Cultured cell lines, although useful for initial non-translational discovery testing, cannot reproduce the complete array of molecular, cellular, neuronal, physiological, and pathological aspects of the mouse model necessary to fully understand the mechanisms investigated in this project, especially regarding ageing and additional co-morbidities. While it is evidently possible to corroborate our data with in vivo studies on human arrhythmia patients, it is not possible to attempt experimental drug rescue on them, creating the need for an animal model. Furthermore, while it is also clearly possible to conduct ex vivo experiments on atrial samples isolated from humans, such experiments would not be translatable to ventricular samples, which are: (1) considerably more difficult to obtain; and (2) unable to maintain an intact arrangement of cardiomyocytes.

This study will be performed using mice because all relevant methods and techniques are successfully established in this species, and because of the availability of genetic alterations in these species. With this approach we will be able to study specific biochemical pathways with a view to understand disease progression. Understanding the effects of interfering with the disease process at specific points in underlying pathways can lead to the development of new treatments for heart disease. All procedures will be conducted by highly skilled, licenced personnel, and non-invasive techniques will be used where possible. Components of procedures will be the minimum required to be consistent with reaching the scientific objectives. Animals will be closely and regularly monitored during the study.

Surgical animals will receive pain relief as standard. Any clinical problems will be dealt with in consultation with the veterinary surgeon. There are strict pre-determined 'humane endpoints' in place, based on clinical signs, at which animals are promptly and humanely killed to ensure that any animal does not suffer unnecessarily.

Which non-animal alternatives did you consider for use in this project?

in silico mathematical models of myocardial conduction human iPSC-derived ventricular and atrial cardiomyocytes

Why were they not suitable?

It should be noted we have considered using our in silico mathematical models of myocardial conduction as an alternative. The basis of such models in the first place is the experimental data collected from our murine model. We then use the computer model to refine our experiments on the GM mice.

Furthermore, since 2018, we have also been developing human models in the form of in vitro experiments on human iPSC-derived ventricular and atrial cardiomyocytes and gradually using them as an alternative to the murine model. However, despite replacing some aspects of the work on GM mice, this in vitro model is largely complementary and thus unable to completely replace our in vivo GM mice model.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The design of individual experiments generally involves factorial design to maximise the information obtained. The majority of measures are quantitative and suitable for statistical

analysis. Comparison between groups will be made by 1-way analysis of variance (ANOVA) or a 2-way repeated measures ANOVA followed by an appropriate post hoc test. We have reviewed a range of published studies that use similar techniques being proposed here and observed great variability in the number of animals used per experimental group. Following our discussions with a statistician, we have calculated our sample sizes for our quantitative experiments by power analysis using a significance level of 5% and a power of at least 80% (Ref: http://dx.doi.org/10.1136/emj.20.5.453).

We will need 8 groups of mice due to:

- 2 mouse genotypes (WT or respective mutant)
- 2 age groups (young or old)
- 2 treatments (control vs treatment with relevant drug – please see details above)

The standard deviation within a group is unknown, hence our power calculations are conducted based on effect size. Aiming for power of 80% and significance level of 5%, an effect size of 1.51 (i.e. the difference between the means of the groups is 1.51 multiplied by the standard deviation) is consistent with 8 mice per group. Based on our previous studies, attrition rates in this kind of experiments is higher in aged mice compared to younger mice. For this reason, we need to add 2 more mice to our calculated n=8/group, thus requiring n=10/group. It should be noted that the exact numbers of animals required will vary with specific experiments as well as the estimates of the coefficient of variation for specific outcome measures; nevertheless, the general principle outlined in the example will be followed. In summary, we need 8 groups of mice at 10 mice/group for a total of 80 mice per readout.

The readouts for each experimental condition include surface ECG recordings, telemetry recordings, patch clamp recordings, and Langendorff recordings. Surface ECG and Langendorff recordings will be performed with the same group of mice, whereas separate groups are required for telemetry and patch clamp recordings. The final number of mice is estimated at 80 mice per readout for surface ECG/Langendorff, telemetry, and patch clamp recordings (total of 240 mice).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have performed pilot experiments in cells to determine a range of appropriate dosage for the drugs we are aiming to use in vivo. Moreover, we have longstanding experience on maintenance and breeding of the appropriate number of animals to maintain the line. Typically, we will always have 2-3 breeding pairs for each line to minimise risks of ‘losing’ the line due to disrupted breeding. From previous experiments, we know when we might need to separate pairs if no experiments are planned. We have also consulted a statistician in our efforts to calculate the appropriate number of animals required in this study. Moreover, as part of our experimental design and good laboratory practice, we will

continuously monitor our experimental needs and re-evaluate the number of mice necessary in our project and data analyses. We will measure production and breeding performance for each line and ensure only the minimum numbers of animals required are bred in any case. Finally, based on previous pilot studies we have optimised serial assessment of electrophysiological measurements and, for example, confocal microscopy experiments to generate data from the same animal, thereby reducing the number of mice necessary in our studies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have recently refined our techniques to include, rather than merely determination of monophasic action potentials and restitution metrics, a multi-electrode array that enables detailed analysis of conduction properties. Importantly, this method is ideal for providing an experimental platform to define not only the role of individual genes in arrhythmogenesis, but also to systematically examine the effects of pro- and anti-arrhythmic interventions on arrhythmogenic substrates.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Animal models: C57BL/6 and S129 mice with mutations such as Scn5a+/- sodium channel haploinsufficiency; deltaKPQ sodium channel gain of function; RyRP2328 ryanodine receptor mutation, Atf5 deletion. The GAA we will use in our experiments have been validated in prior experiments and they are useful models for the project.

Methods include breeding and maintenance, ECG recordings, telemetry, patch clamp, Langendorff, drug administration.

Why can't you use animals that are less sentient?

In principal, mice, despite having a smaller heart that works at much higher heart rates compared to humans, share considerable genetic homology and their hearts have similar cardiac conduction systems to humans. This justifies using mouse models in studies of arrhythmogenic phenomena.

Furthermore, genetically modified murine cardiac models potentially fully replicate the genetic changes underlying congenital clinical conditions associated with arrhythmogenesis, without requiring prior pharmacological intervention – we have previously developed a range of monogenic mouse models in our previous Project licences (Scn5a+/- sodium channel haploinsufficiency; deltaKPQ sodium channel gain of function; RyRP2328 ryanodine receptor mutation) and will now use these models, as well as other commercially available GM models in this study. These mice make powerful drug testing models to explore the cardiac electrophysiological events associated with drug administration, as well as the response mechanisms particularly in arrhythmic conditions.

Using a transgenic mouse model will allow us to continue delving into the mechanisms by which genetic alterations coincide with complex disease fostering a proarrhythmic environment. No other species less sentient is available at the moment that will allow obtaining these results.

Large animals models are not suitable for this study when it comes to ageing as they require longer durations to age and thus lead to more welfare implications. Whilst pigs maybe an option (ignoring ageing component) the preclinical perspective is not entirely captured by the pig model. An equally valid and suitable GM model is not available in pigs and the mouse model purposed offers also the advantage of being already a validated model for these conditions and drug testing.

Given the well-established contributions of sedentarism, obesity, diabetes mellitus and metabolic syndrome to the presently increasing prevalence of arrhythmic phenotypes in the UK population, such an opportunity is of great relevance to the future clinical treatment of NHS patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals used in the experiments described herein are not expected to exhibit any harmful phenotype. For older animals, special consideration will be given to their environmental enrichment. Enrichment for aged mice will follow best-practice guidelines as recommended by the Experimental Biology Unit. We will be monitoring our mice on a daily basis. After initial recovery from surgery, adverse effects may include wound infection, which is extremely rare. We will prevent that using good aseptic technique in our procedures, perioperative analgesia and implementing a monitoring regime. In the event of any animals showing any signs of mild discomfort (e.g. swelling, redness or discharge at the operation site), but are otherwise well will treated by minimally invasive methods following advice from the NVS. The animal will be culled by a Schedule 1 method if no improvement is observed within 24 hours of treatment. In all cases, we will work closely with the NVS and seek advice on animals whose welfare is giving reasons for concern. We will use gas anaesthesia which is safer than injectable anaesthesia and we will monitor our animals during anaesthesia and keep them on heating pad. Whenever possible we will try to carry out more techniques during the same anaesthesia event to reduce them (ex.

Imaging, drug administration, sampling during the same anaesthesia). We have implemented a specific welfare monitoring method for ageing signs and a details scoresheet with actions to better assess old ageing animals welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The most effective means of elucidating the function of genes whose perturbations are implicated in cardiac arrhythmogenesis is the integration of a GM mouse model with other laboratory experiments. Mice lines with defined and well characterised genetic background such as Scn5a+/- sodium channel haploinsufficiency; deltaKPQ sodium channel gain of function; RyRP2328 ryanodine receptor mutation; PGC receptor mutation, Atf5 deletion are readily available and suit the work of this project. The mouse lines chosen have extensively been validated and have previously been successfully used to recapitulate the human condition thus proving to be a superior translational model. All lines used in our work will thus conform to such rigorous selection ensuring that only the most reliable and translationally relevant modules are utilised.

All the mice will be protected against any potential infection as they will all be maintained in a full- barrier clean environment in our recently opened state-of-art new Biomedical Research Facility.

Environmental enrichment will provide good living conditions to the mice and reduce the stress and mice will be checked at least once daily. Enrichment for aged mice will follow best-practice guidelines as recommended by the Experimental Biology Unit. All procedures will be performed by trained and competent persons and following NVS advice. We will perform ECG which are well characterised procedures carried out routinely and very well tolerated in rodents. To minimise stress ECG will be carried out under anaesthesia following NVS advice and animals will be checked after the procedures. We have extensive experience (15 years) in ECG recording and experienced problems in less than 2%.

Animals undergoing surgery may experience transient post-operative pain or discomfort. Anesthesia and pain management regimes will be administered upon advice from our NVS. Adequate analgesia will be provided at the beginning and after the surgery to minimize animal suffering. We will provide particular care for recovery after surgery and will include an animal welfare scoring sheet for each animal for health check. If the levels of pain, suffering or distress exceed severity limits indicated by the protocol, we will take advice from our NVS and, if necessary, sacrifice the animal with a Schedule 1 method.

Our animal technicians and NACWO are experienced in management of animal population health and welfare. We also have extensive experience (6 years) in surgery to implant telemetry and have rarely experienced any adverse effects (less than 5%). Surgery for implantation of telemetry device is performed under aseptic conditions and has a moderate severity limit. In addition, this surgical procedure is a well-characterized technique done

routinely by our group and the implant is well tolerated by rodents. Mice will be euthanized by schedule 1 methods or under terminal anaesthesia under protocol 1,2,3,4 and, whenever reaching humane endpoints detailed in the protocols, with a Schedule 1 method.

Animals receiving drugs treatment will be monitored appropriately and whenever possible oral route of administration will be favoured to parenteral routes. Drugs tested, including their dosage and length of administration, will be selected mainly among well characterised drugs and their use will be preceded by literature review and eventual previous in vitro tests. We will use single use needles for all our injections and avoid tail handling our mice.

Animals will not be single-housed and the last mouse in a cage will be used within 1-2 days after its last cage mate is taken. The study design has included comparison between knockout and wildtype littermate controls which will reduce the biological variation and increase the sensitivity, reducing the number of animals needed.

In carrying out our work, we aim to use older mice with florid phenotypes in order to minimise their suffering and the total number of GM and WT mice in use.

No severe protocols will be used.

Finally, we will adhere to the ARRIVE guidelines in regards to the reporting of research involving animals.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will be checking the literature for 3Rs advancements regularly and implement these accordingly. In addition, we will be working closely with the NACWO, NIO and NVS, as well as engage with the University’s User Forum and latest guidance from the NC3Rs on recent and general advances in the 3Rs.

140. Modelling gene therapy for congenital bladder dysfunction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

therapy, congenital, urinary, genetic, neuro-muscular

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim is to take the first experimental steps to cure congenital diseases that affect the nerves and muscle of the bladder and prevent normal urination. We will gain biological insights into these diseases by studying genetic mouse models. We will use these models and the biological insights as a platform to test whether gene therapy can safely cure these diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Nearly 30 in 10,000 live births have congenital anomalies of the urinary tract, the 3rd most prevalent anomaly in the UK, and one of the commonest disorders detected when prenatal ultrasound screening is performed. Some severely affected foetuses undergo termination while others are born. Of those born, many suffer from kidney failure as children or adults, and require dialysis or transplantation, without any cure for the underlying pathology. One particular subset of anomaly affects nerve – muscle communication and as such causes functional, rather than anatomical, obstruction of the urinary tract.

In the last 25 years, genetic causes of many congenital diseases have been defined, opening the possibility of developing cures that target the underlying molecular pathology. Effective cures for diseases with a genetic basis are being sought in other disease systems, utilising understanding of the genetic and biological disease pathology, for example in Spinal Muscular Atrophy. Here, modified viruses are used to deliver therapeutic genes to the affected tissues. This has not been attempted for diseases that affect the urinary tract, despite the associated health burden and an increasing understanding of the genetic bases for these diseases. Neuro-muscular diseases that affect bladder voiding are a promising target for gene therapy as there is no anatomical anomaly per se, opening the possibility that by resolving the underlying defect at a gene level we will be able cure the disease. To establish whether gene therapy could be efficacious it is essential to fully understand the disease processes, and how these change with age (the disease natural history). We will use this knowledge to design and test therapeutic strategies in animals that have the disease. This will pave the way for evaluating the safety and efficacy of therapies in people with congenital voiding dysfunction.

What outputs do you think you will see at the end of this project?

This work will demonstrate the feasibility of using gene therapy to treat mouse models of bladder voiding dysfunction. Such studies have not been performed previously in the urinary tract and the results will form the basis of multiple high impact publications. Totally novel information will be obtained on the feasibility of using viral vectors to deliver therapeutic molecules, and treatment parameters will be defined. Crucially, the efficacy of using viral vector-delivered gene therapy to prevent congenital bladder dysfunction will be determined. If the pre-clinical trials described in this licence are successful, they will pave the way for use in human clinical trials.

Who or what will benefit from these outputs, and how?

There is no cure for congenital bladder dysfunction, and affected individuals are at a high risk of renal failure and death, with costly continual treatment. Therefore, there is an urgent need to develop new treatments. In the short term, these studies will set a precedent for

how advanced therapies, such as using viruses to deliver gene therapy, can be used to treat animal models of voiding dysfunction. This will be of great interest to various parties, including urologists, researchers that study the urinary tract, and researchers who are involved in the application of advanced molecular therapeutics.

From a patient perspective, these studies are designed to lead to clinical trials and as such will be of potential direct benefit to affected individuals. Here, we can look to the precedent set in the treatment of spinal muscular atrophy, where successful preclinical trials of viral vector mediated gene therapy were run in genetic mouse models of the disease. This subsequently led to human trials and regulatory approval in USA and EU in under eight years, with treated children effectively cured.

From the perspective of policy makers and funding bodies, this work will demonstrate another disease system in which gene therapy can be efficacious. We are working closely with large program bids to attract funding for Rare Disease research, and this work will contribute to the relevance of ‘personalised medicine’ in treating rare and costly diseases.

How will you look to maximise the outputs of this work?

Our results will be published in original research papers in high impact journals. We will report ineffective therapeutic strategies as well as the effective strategies, an essential step in the development of novel therapeutics. We will also discuss our work at international conferences, such as the International Continence Society, and we have collaborations with both gene therapy and urology experts.

Species and numbers of animals expected to be used

- Mice: 4000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Urinary voiding is a complex, multi system behaviour. Mice model many of the physiological aspects of human voiding, and there are genetic mouse models for diseases that affect bladder voiding, including a number with a neuro-muscular basis. It not possible to model the full range of physiological defects and therapeutic effects in non-mammalian systems. We will study embryonic and postnatal to adult mice, to identify the developmental origin and natural history of the diseases being studied. This will inform our therapeutic strategy by indicating a therapeutic window. In addition, we also require adult mice to maintain our genetic mouse colonies.

Typically, what will be done to an animal used in your project?

Mice will be bred from parents that each have one copy of a mutant gene, resulting in ¼ offspring with both copies of the mutant gene. These mice will have bladder voiding dysfunction. Within 48 hours of birth, the mouse will receive an injection of the virus containing the therapeutic gene, into the superficial temporal vein. The therapeutic administration causes no lasting harm to the mouse, but can involve anaesthesia. The mouse will be observed and analysed by non-invasive techniques, such as ultrasound or voiding behaviour evaluation for up to eight weeks. The mouse will be humanely killed or will undergo a physiological technique to measure bladder function, while under terminal anaesthesia, and then be humanely killed. Some mice that are not used to model a therapy will be studied for up to one year to understand the underlying disease mechanisms. While alive, they will be studied with the same non-invasive techniques and then humanely killed or analysed for bladder function while under terminal anaesthesia.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will be bred from parents that each have one copy of a mutant gene, resulting in ¼ offspring with both copies of the mutant gene. These pups will have bladder dysfunction and may develop mild urinary tract malformations, which is compatible with normal health. There is a small possibility of significant dysfunction developing, which would put mice at risk of developing kidney failure. While this condition is not painful it does cause ill health. Accordingly, mice will be monitored from birth and those showing persistent signs of ill health will be humanely killed, to ensure the project does not exceed moderate severity. Homozygous mutants in our established Hpse2 and Lrig2 mutant lines show slower weight gain compared to their littermate controls. We will monitor litters for signs of distress and weight gain, and cull any mice that show signs of distress or abnormal behaviour, or if weight gain is sufficiently slow that they approach 50 % weight of littermate controls.

Pups up to two days old will be treated by injection of therapeutic molecules into the superficial temporal vein in the head. This route of delivery does not cause any lasting harm to the mouse, but there is a risk of maternal rejection when the pup is returned to its mother, and there is risk associated with anaesthesia, if used. To minimise this risk we will allow the mouse to ‘pinken’ on a warmed pad before return.

The viral vectors we use have been extensively studied in animal models and have been approved by the EU and the US FDA for clinical use. They are non-pathogenic, are engineered to be replication and integration deficient, and the risk of an auto-immune response is exceedingly small in laboratory animals. The therapeutic molecules that will be delivered by the viral vectors are widely expressed in wildtype mice and each transgene will be individually assessed for risk in the relevant GMO form.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The heterozygous with wildtype breeding animals are all expected to have mild severity; offspring of heterozygous with heterozygous parents may experience moderate severity depending on their genotype. Treated mice will also experience a moderate severity. Overall, 2/3 of animals will experience a mild and 1/3 a moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Voiding is a complex, multi system behaviour. Although there is considerable data on gene expression patterns in the organs of the urinary tract these are not sufficient to allow an understanding of the anatomical and physiological perturbations that are caused by mutations in these genes and it is not possible to model the full range of physiological defects and therapeutic effects in non-mammalian systems. Given the need for realistic preclinical models, there is currently no alternative to using live animals. Rodent urinary voiding models many of the physiological aspects of human voiding, and there are faithful genetic mouse models for many diseases.

Which non-animal alternatives did you consider for use in this project?

We have used cell lines, including nerve cells and muscle cells, to model specific aspects of the genetic diseases and test the therapies described in this project. For example, we test the ability of each viral vector to get into the cell and drive expression of the therapeutic gene before we use the vector in the animal model. Viruses are applied to cells in culture and the amount of gene being produced will be assessed quantitatively, and visualised to see if it is in the expected part of the cell.

Why were they not suitable?

Cell lines and other in vitro techniques do not model the complex interplay between multiple tissue types that is necessary to understand these genetic diseases. Delivery of therapeutics to cell lines gives some indication of the potential efficacy of the strategy but do not offer further insights into how these technologies will behave in the complex environment of a living animal. For example, it is also not possible to model an immune response in vitro or how other organs will be affected. It is essential to gain knowledge on all of these aspects of the disease and the therapeutics as the fundamental aim of the project is to bring these technologies into the clinic to treat patients.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Extensive experience with animal models in previous similar projects indicates that 2000 animals will be required for five years to maintain the multiple colonies used in this project, while a similar number will be required for the specific experimental protocols.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use non-invasive techniques to obtain a large amount of data for each mouse. We will use scans to monitor the structure of the renal tract, which require anaesthesia but are minimally invasive and can be used recurrently on a single mouse to monitor disease. We will analyse voiding behaviour with the voiding stain on paper technique, which simply involves placing a mouse on blotting paper for some hours then imaging the urine stains, but gives a lot of information on the ability of the mouse to void.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Each experiment has been assessed by to determine the optimal number of mice. We have considerable previous experience and data from animal models to do this accurately. Our group also has great experience in efficiently running mouse colonies to ensure the minimal required numbers of mice are bred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The models we will use are faithful genetic models of human voiding disease, and as such will themselves have urinary voiding dysfunction. The primary defects do not cause

significant distress to the animals and we will humanely kill any animal that displays signs of secondary disease, such as kidney disease. We use non-invasive techniques multiple times on individual animals to maximise the data gathered from a single animal, without causing harm. We have refined our techniques in line with changing good practice and our own expertise, for example in regards to the superficial temporal vein injection into neonates. Originally, we used both anaesthesia and ice-induced hypothermia. Along with our colleagues, we have determined that the ice is not necessary to immobilise the mice and we have removed this step from the protocol. We will receive training to develop our pup restraint technique to not require anaesthesia, as this bears some risk to the pup. In addition, as our understanding of the therapeutic vector efficacy develops, we will further refine this protocol by selecting only the mutant mice and appropriate controls for treatment - this will require tattooing pup foot pads, taking tail clips from the neonatal mice and rapidly genotyping the whole litter.

Why can’t you use animals that are less sentient?

To accurately model the efficacy of therapies it is essential to use animals with mature voiding function that is similar to adult human voiding. We have intensively studied Hpse2 and Lrig2 mutant mice and confirmed they have bladder voiding dysfunction similar to that seen in human patients with urofacial syndrome (other groups have studied mice with mutations in Chrm3 and ChrnA3 and also identified bladder dysfunction). In addition, there are no non-mammalian species that provide a sufficiently faithful model of normal human voiding, or of human congenital bladder disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will stay informed of updated and variant protocols being used in the scientific community, such as best practice for temporal vein injections, by reading the literature and discussing at meetings.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow LASA guidelines and NC3R guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly check the NC3R website for changes in best practice advice, and engage with the animal facility for guidance on other advances.

141. Metabolism as a novel target for cancer therapy

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Cancer, Metabolism, Therapy

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand what factors determine how tumours metabolize different nutrients and how to manipulate these factors in order to develop more efficient anti-cancer therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The abnormal metabolism is one of the well-known hallmarks of cancer. Tumours have been demonstrated to consume more of various nutrients, including glucose and glutamine, and catabolise them differently from normal tissues. Different types of cancers can be stratified based on the expression of metabolic enzymes and genes encoding them, which can predict the aggressiveness of the disease and the therapeutic outcome. The catabolism of major nutrients through specific metabolic pathway in tumours fuels the synthesis of molecules vital for proliferation and survival of cancer cells as well as their ability to metastasize. Metabolic enzymes regulating these pathways in tumour cells have been proposed as plausible therapeutic targets. However, metabolism of tumours and their metabolic requirements and dependencies are determined by multiple factors including genes driving cancer, the tissue a tumour originates from and the interaction between tumour and non-tumour cells. We will evaluate how this interaction between genetic and non-genetic factors determines how tumours metabolise different nutrients and what nutrients and metabolic pathways tumours depend on. With this we hope to be able to dissect the complexity of tumour metabolism in order to understand how to target it for therapeutic benefit.

What outputs do you think you will see at the end of this project?

In this project, we expect to advance our knowledge of how cancer and normal cells metabolise different nutrients and how these processes are being regulated. We will share our results with other researchers in the form of peer-reviewed original research articles and reviews in specialised scientific journals as well as, potentially, in cancer biology book chapters. We also aim to develop novel cancer models, other technical resources such as protocols and analytical tools for cancer and metabolism analysis and, importantly, novel anti-cancer therapies.

Who or what will benefit from these outputs, and how?

Clinical and academic research scientists and students of cancer biology would benefit from the knowledge/resources generated in this project. The project will also facilitate the identification of key metabolic specializations between different organs in health and disease. Novel therapeutic targets may be discovered for developing new drugs. Therefore, ultimately patients with various types of cancer may benefit from these novel treatments.

How will you look to maximise the outputs of this work?

We aim to disseminate our findings to scientists in our own and other fields, as well as more widely in the public domain, by presentations at national and international meetings and publications in high- impact journals.

Species and numbers of animals expected to be used

- Mice: 26,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project is focused on contribution of major oncogenes disregulated in human cancers and tissue environment to metabolic changes observed during tumorigenesis in vivo to identify novel therapeutics for cancer patients. Therefore, we will need to use adult mice as a model for our project, because they have shorter latency of tumour development compared to higher vertebrates, yet their physiology, anatomy, metabolism and pathological manifestations better resemble those in humans compared to the features of other commonly used animal models, such as nematode (C. elegans), fruit fly (Drosophila melanogaster), or zebrafish (Danio rerio).

Typically, what will be done to an animal used in your project?

Most of the animals used in this project will develop primary tumours of the internal organs, such as the lung, liver, mammary gland and intestine. These primary tumours may metastasise to other parts of the body, such as the brain, liver or lung, causing formation of secondary tumours, as it happens in human cancer. The tumours will either appear spontaneously due to inherent genetic alterations or be induced by the administration of either viruses or plasmids that will carry either enzymes or genes enabling such genetic alterations or by a transplantation of cancerous cells or tissue pieces.

After tumour induction, animals will undergo different experimental procedures. Animals may be sampled for small portions of blood to monitor changes in various biochemical parameters related to cancer development. We may treat animals with chemical substances that switch on or off the function of some genes or proteins, that could be relevant to tumour growth, or with labelling agents that would facilitate visualisation of certain types of cancer cells or cells interacting with the tumour. Animals may be placed on modified diets lacking certain nutrients. The control animals in this case will be placed on artificial diets with controlled complete composition. Animals may undergo imaging to monitor tumour growth by using both modalities that are used in humans, e.g., ultrasound, MRI or PET, and by state-of-the-art specific animal imaging approaches based on luminescence and fluorescence measurements. Most of the animals as well as control counterparts, which will not have tumours or will not undergo the abovementioned procedures, will be culled by approved humane methods and their tissues will be dissected and used in experiments in vitro to examine the molecular and cellular mechanisms of the processes driving cancer growth and metabolism.

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the experimental animals in our project will develop some forms of tumours. In general, tumours in mice either emerge spontaneously or are triggered by injection of viruses or plasmids that will carry either enzymes or genes enabling genetic alterations and or by transplantation of malignant cells. In mice genetically predisposed to develop cancer spontaneously, tumours typically develop in the period between 3 to 6 months; in some cases, up to 12 months. Depending on the site of the tumour development and its stage, animals will experience different adverse effects. Animals with liver tumours may develop anaemia, jaundice and ascites (abnormal build-up of fluid in the abdomen). Animals with lung cancer may be asymptomatic initially, but after several months may develop breathing difficulties and anaemia. In addition, a proportion of the lung tumours may be highly metastatic and in the end stage, lung cancer may be accompanied by disturbances in other organs affecting animal well-being. In animals with spontaneously emerging (autochthonous) tumours, these side effects typically occur at the end stage of tumour development, gradually developing over 1–2 months.

For transplantation models, the time frame between tumour transplantation to the humane endpoint is shorter, typically between 1 and 2 months. Some of the adverse signs mentioned above for spontaneous cancer models will therefore develop over a shorter period of 1–2 weeks. In addition, mice undergoing transplantations may be impacted by the effects of injections, which could cause local haemorrhage and damage to the organs being injected.

In all cases, tumour burden will be limited to the minimum required for a valid scientific outcome. Animals may display tumour ulceration, labored respiration, or persistent diarrhea, but they will be immediately culled after any of these symptoms is observed. The animals will be also monitored for weight loss and body condition score, and mice dropping below the set criteria (e.g., 15% weight drop) will be immediately culled to prevent excessive suffering.

Animals that will undergo surgical procedures may experience mild to moderate pain immediately after the surgery and they will be given analgesics to minimise post-surgical pain.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

For the majority of mice that will be developing tumours in the course of the project and/or will undergo surgeries (80% in total) the severity will be moderate. Approximatly 20% of these animals will be used for tumour and other tissue collection after being killed by a Schedule 1 method or after a non-recovery procedure under general anaesthesia (approximately 20%). The rest will be either administered different gene/protein modifying or therapeutic agents, and/or placed on modified diets or subjected to food restrictions. In all of the cases the overall severity will not exceed moderate level. For the rest of

the animals, which can be either genetically modified but without any obvious clinical signs or wild type and/or receiving infrequent injections, the severity will be mild.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

To identify mechanisms of tumour growth and metabolism in physiological condition, which are meaningful for human clinical studies, we need to use animal models because they develop tumours similar to human ones. In addition, the metabolic interactions between tumour and a host as well as effect of local and systemic effect of dietary manipulations can only be meaningfully studied in vivo.

Which non-animal alternatives did you consider for use in this project?

Evidence will be collected from initial studies using in vitro cell culture /organoids/tumourspheres model systems, where appropriate, as well as by surveying the mammalian and other literature. In addition, we will use non-regulated procedures to collect expression data from fixed non-GM mammalian tissues and functional/expression data from genetically and/or environmentally manipulated in vitro system.

Those generated data sets will be analysed by bioinformatics, and thus to identify the most promising candidates to be validated in experiments in vivo.

Why were they not suitable?

Organ- and cancer-specific metabolic pathways are highly influenced by nutrients, oxygen, circulating hormones and other aspects of the complex physiological environment inside the body. It is not yet possible to recapitulate all of these parameters in vitro, nor to mimic the metabolic crosstalk between different organs and tumours –a key aspect of this research project.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers were estimated based on our previous experiences, statistical modelling, and predictions for exploratory experiments. For spontaneously occurring tumours (autochthonous models), due to the high variability in tumour progression, for each cohort we normally would need around 20 animals per group for study, whereas transplantation models would normally need between 10–20 animals per group.

We will take as an example an experiment where the requirement of a metabolite both produced by a biosynthetic pathway and available through a diet is being evaluated for the progression of tumours. In this case animals are either administered an inhibitor of the biosynthetic pathway producing the metabolite or place of a diet lacking this metabolite or a combination of both treatments is being used. Considering these potential treatment combinations, each experiment would normally need at least four groups: control/vehicle treated/control diet; metabolic inhibitor/control diet; vehicle treated/experimental diet; metabolic inhibitor in combination with an experimental diet. Furthermore, for all novel therapies, the dosing frequencies and drug dosage needs to be optimised first. There are also at least two different regimes: prevention and intervention. In the liver model we will test these combinations in the inducible liver models and in the model induced by hydrodynamic transfection (3 oncotypes per each model): (20GEM + 10 hydrodynamics) x 3 oncotypes x 4 experimental groups x 2 designs = 720 animals. For 3 treatment combinations we will need 2160 animals (Metabolism of Liver Tumours).

Additionally, to evaluate how our findings would translate in human cancers we will use xenograft models. For 5 xenogfrats we will perform treatment combination experiments: 5 x 10 mice per group x 4 experimental groups x 2 designs = 400 mice. For 3 treatment combinations we will need 1200 mice.1200 = 1200 mice with xenografts.

For these experiments we mostly plan to use mouse models of the two major cancers: mammary gland and liver, comparing at least 3 oncotypes per each type.

As mammary gland tumour models, we will also propagate 15 breast cancer PDXs and will perform initial multiomics analysis (10 animals for propagation + 10 for analysis): 20 x15 = 300. For 5 PDXs we will perform treatment combination experiment: 5 x 10 mice per group x 4 experimental groups x 2 designs = 400 mice. For 3 treatment combinations we will need 1200 mice. 1200+300 = 1500 PDX mice. Some of these PDXs will be propagated and used in the experiments with fat pad clearance.

Altogether, we will use 2160 GEMM animals and 1200 animals with xenografts for the metabolism of liver and mammary gland tumours each, In addition, 1500 animals with breast cancer PDXs. 8220 animals altogether.

In addition, we will run pilot exploratory experiments with smaller groups of animals in lung and intestinal tumours, probing different approaches to study the relationship between metabolism and tumours. We will need approximately twice fewer GEMM animals in this initial phase (2,200 in total). Therefore, we will need 8220 +2200 = 10420 mice in experimental protocols, out of which 6520 are GA animals.

Considering that some animals may not develop tumours or may be excluded from the subsequent experiments due to various reasons before overt tumour development, as well as taking into account that some animals will have to be initially generated from breeding of GA mice, where not all progeny will be suitable for experiments (some genotypes can be 30-25%), we would need to produce on average 3×6,520 = 19,560 GA animals for the programme of experiments. For those we will need 1222 breeders (8 mice per litter, 4 litters per breeding pair). Altogether – 20,782. Some of the breeders will be on doxycycline inducible diet (200). Finally, we envisage that we may need to generate new genetically altered lines of mice or import some novel genetically altered animals from other laboratories. For this purpose, we will need 1500 animals. Therefore, we plan to use 3,900 (PDXs/xenografts) +19,560 (GA) + 1,222 (breeders) + 1,500 (new strains) = 26,182 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The efficiency of animal usage is maximised in consultation with animal technicians by careful control of breeding to match research needs with respect to numbers, phenotypic uniformity and health. In addition, we will also take advantage of the online tools, including the NC3Rs Experimental Design Assistant to help us with experimental designs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

This programme of work will make optimal use of several tissues, fluids and cell types per individual mouse. We will aim to collect organ samples from multiple body sites and to provide other affected tissues to appropriate scientists, so that they do not have to breed mice specifically for their experiments. This highly integrative approach will maximise the information obtained from the minimum resources. Cryopreservation of gametes, embryos, tissues and cells is a routine procedure and will ensure that the minimum number of mice is bred.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been selected for the majority of this work as it is an appropriate model for providing insights into human diseases and it is the species in which reliable transgenic and knockout technologies are most advanced. Wherever appropriate, we will minimise the adverse effects associated with genetic alterations by using inducible or conditional alleles to delete gene activity from specific tissues rather than from the entire mouse.

Genetic alterations or transplantation methods will be used to induce tumour formation. These models possess relevant pathological features similar to those in human patients, allowing us to investigate basic tumour biology and to perform preclinical trials. This will obviate the need to use higher vertebrates. Furthermore, whenever possible, we will perform experiments in transplant models, which usually exhibit a faster disease course, thus minimising the duration of distress and lasting harm to the animals. We will only perform key experiments in the autochthonous settings, particularly when we will need to elucidate how tumours develop, evolve, and interact with their native microenvironment.

In all surgery, analgesia will be provided according to contemporary best practice and advice from the NVS/NACWO. Good aseptic surgical techniques, heat & fluid therapy will be provided as necessary. In the case of cancer models for example, we will follow the guidelines in Workman et al, British Journal of Cancer (2010) 102, 1555 – 1577.

Whenever possible, we will use terminal anaesthesia to perform surgical experiments in animals to decrease their pain and suffering.

Why can't you use animals that are less sentient?

This project is focused on understanding the role of metabolism in tumourigenesis in vivo to identify novel therapeutics for cancer patients. Therefore, we will need to use a model system that is able to establish tumours and has metabolism closely resembling that of human. Therefore, mice would be the most appropriate animal model for our project. Less sentient animal models, such as nematodes or flies, will not be suitable for our purpose. Whenever appropriate, we will carry out experiments in terminally anaesthetised animals, but for some of the proposed experiments, we will investigate various methods to intervene with the tumour progression. In such cases, we will need to observe the animals over a prolonged period of time until the assigned endpoints are reached.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For all manipulations we will adhere to local or national guidelines that aim to minimize suffering. Many of the genetic and dietary manipulations as well as the administrations of gene inducers/repressors or other agents are standard and previous refinements from the literature will be used. If, however, there is insufficient information available, new manipulations will be pre-screened in small-scale pilot studies to obtain indications of the minimum dose and exposure time that is likely to be effective, thereby minimising any potential suffering.

We will also consult our NVS to improve the surgical methods and procedures we are using.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experiments will be planned in accordance with the recently formulated PREPARE guidelines.1 For surgical procedures, we will follow the guidelines set out in LASA Guiding Principles on Preparing for and Undertaking Aseptic Surgery. With regards to the experiments in cancer models, we will adhere to the Guidelines for the Welfare and Use of Animals in Cancer Research2 and will monitor body condition according to system developed by Ullman-Culleré and Foltz3. In addition, I will also follow the latest advancements in relevant fields, by attending conferences, reading journal articles, and collaborating with experts in these areas, to ensure that the experiments will be conducted in the most refined way.

1. Smith, A. J. et al. PREPARE: guidelines for planning animal research and testing. Lab Animals 52, 134–141 (2018).

2. Workman, P. et al. Guidelines for the welfare and use of animals in cancer research. Br J Cancer 102, 1555–1577 (2010).

3. Ullman-Culleré M.H. and Foltz C.J., Lab Anim Sci. 1999;49(3):319-23

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will keep following up the latest publications in the fields as well attend courses and seminars and follow NC3Rs and NORECOPA websites to learn any advances in the 3Rs.

142. The role of morphological and genetic abnormalities in tumour progression

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

cancer, tumour microenvironment, centrosome amplification, tumour evolution, chromosome instability

Animal types	Life stages
Mice	neonate, juvenile, adult, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims at investigating how well-established alterations that occur in cancer cells, and not in normal cells, impact the architecture of tumours, what surrounds them and how they progress.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Many cancer-associated abnormalities cause irreparable stress and are thus poorly tolerated by normal non-cancer cells. However, cancer cells inevitably find ways to cope with these aberrations. Understanding how these specific cancer-associated traits impact tumours will allow us to establish and explore survival mechanisms employed by cancer

cells to cope certain abnormalities. This is key to develop better therapies that aim at targeting these coping mechanisms to selectively kill cancer cells.

What outputs do you think you will see at the end of this project?

At the end of this project, we will have generated new information regarding how cancer-associated abnormalities impact how tumours progress, how it modulates the tumour architecture and surrounding environment and how these changes affect tumour progression. The new information generated will be made available to the scientific community via publication in peer reviewed journals.

Who or what will benefit from these outputs, and how?

In the short-term duration of this project (up to 5 years) the main benefactors of this work will be scientific community that will have access to our research through publications and presentations. This project will provide valuable information about how cancer cells respond to specific cancer-associated abnormalities and how this affects how cancer cells progress, metastasize and change their surrounding environment.

In the long-term, after the completion of this work, the information obtained has the potential benefit cancer patients as follow:

• Development of biomarkers for disease progression and response to therapy by whole exome sequencing of tumours collected in this study. Some cancer-associated abnormalities can be associated with poor prognosis and disease progression. Furthermore, these abnormalities frequently require cancer cells to adapt in order to effectively grow and divide. These adaptations can indeed be targeted, making these abnormalities attractive for the development of specific therapeutic strategies. Thus, defining signatures to identify these abnormalities could lead to the development novel prognostic biomarkers and to define appropriate treatment options.

• Development of novel therapies that target changes in the tumour surrounding environment induced by cancer cells carrying specific abnormalities. Despite recent progress, it is clear that novel and selective therapies that kill cancer cells are needed. We hypothesise that how cancer cells adapt to and grow in the presence of specific abnormalities is an important part of their survival requirements that could be targeted. We will explore if changes induced by these abnormalities are important for their survival and adaptation and could be used to develop novel therapeutic strategies.

How will you look to maximise the outputs of this work?

It is important to maximise the outputs of our studies, more so if the studies involve animals. To do so, we have in place the following strategies that will be implemented:

- Make data available through Open Access peer-reviewed publications will ensure that our work reaches the wider scientific community.

- All proteomics and sequencing data obtained from this work will be made available in appropriate public repositories: PRIDE (proteomics; http://www.ebi.ac.uk/pride/archive/), Arrayexpress (RNA-seq data; https://www.ebi.ac.uk/arrayexpress/).

- Dissemination via talks and posters at scientific conferences will allow our work to reach scientific community before its publication.

- Dissemination in the context of 3Rs, for example NC3R meetings and workshops, which will allow us to share our findings and keep up with best practices to improve our animal work.

- Establishment of collaborations, for example with scientist clinicians that could help translation of our work to the clinic.

- For most of our animal tumour experiments, we maximise outputs by removing other tissues of interest that could be shared with colleagues to develop new projects. For example, we propose to remove lungs, mammary gland, ear, tail and lymph nodes.

Species and numbers of animals expected to be used

- Mice: 8500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Our goal is to develop a better understanding of cancer progression, dissemination and evolution in order to develop therapeutic strategies that can be translate to the clinic. The mouse has many

characteristics that makes it is an ideal model for our studies:

- Mice have been used successfully for decades by scientists to understand tumour biology and develop effective drugs in the clinic.

- Mice have a breeding-cycle that is relatively quick.

- Many genetically altered (GA) mice are available to make scientific research efficient, relatively quick, and require fewer animals (no need to generate some of the models that already exists). The technologies to generate further GA mice that possess more relevant gene modifications is now routine and provided through commercial sources which limits the errors by less experienced staff in making these animals.

Because we are interested in cancers that occur mostly in adult humans (breast, pancreatic and ovarian cancer), we will use adults and aged animals. For genotyping, which may need to be done early, we can also use neonates and juveniles.

Typically, what will be done to an animal used in your project?

- All experiments require a pre-planned series of procedures that are designed to maximise the data from the use of the mice, while using the minimum number of mice and imposing the minimum amount of discomfort and harm.
- Some experiments may require mice to undergo repeated anaesthesia, often firstly for a minor surgery to allow implantation of cells. Most therapies that require repeated administrations are given via IP or SC as this seems to be give minimal discomfort and results in rapid accumulation of the compound in the circulation. For most injections mice are not anaesthetised, as experienced staff do each injection in a few seconds removing the harm of additional anaesthesia. Repeated tail vein injections are avoided as much as possible as tails can be become inflamed and scabbed with the repeated wounding with a needle.
- We use many types of imaging to monitor the progress and response of tumours and ensure they will not exceed the limits allowed for tumour size/volume. These require mice to be anaesthetised and injected with either a substrate (luciferin) or a radiotracer (for SPECT or PET studies). MRI does not require an additional injection but does require more skill in interpreting the resulting images, so this will be factored in when decided what is the best approach.
- A common experiment design for mammary tumours might be: tumour cells are implanted in the mammary fat pad (week 1), this requires surgery and anaesthesia. Tumour presence/growth can be evaluated by palpation. Tumour growth and dissemination is monitored by imaging (e.g. BLI) once weekly (weeks 3-8). Mice are terminated as tumour volume (established using either mm3 determined MRI volumes, calibrated BLI signals from the same MRI analysed mice or using callipers) reaches 1250mm3 total tumour burden or mouse welfare suggest earlier termination.
- A common experiment design for mammary tumours undergoing therapy might be: tumour cells are implanted in the mammary fat pad (week 1), this requires surgery and anaesthesia. Tumour presence/growth can be evaluated by palpation and therapy commences (week 2-3), therapy is repeated 2-3 times (if IP) per week or once daily for a maximum of 7 days if by oral gavage (weeks 3-5). Tumour growth and dissemination is monitored by imaging (e.g. BLI) once weekly (weeks 3-8). Mice are terminated as tumour volume (established using either mm3 determined MRI volumes, calibrated BLI signals from the same MRI analysed mice or using callipers) reaches 1250mm3 total tumour burden or mouse welfare suggest earlier termination.

What are the expected impacts and/or adverse effects for the animals during your project?

- Injections give transient pain as they do in humans and mice recover rapidly.

- Mice recovering from surgery will receive analgesia and usually show little change in their general behaviour, mobility, feeding or drinking suggesting the discomfort is manageable.

- Early tumour growth does not usually cause pain or discomfort. As tumour grows, it is possible that its location may cause physical problems (in eating, mobility) or it may become ulcerated. In most cases these mice will be immediately killed by Schedule 1 unless advice from the NVS suggests the discomfort can be managed with appropriate drugs.

- Implanted tumours tend to develop and grow at a more predictable time frame that GA mouse tumours. Thus, GA mice experiments often require several months before tumours arise and experiments can be conducted whereas implanted tumours are often completed in 3 months.

- If the weight loss reaches 15% the mice will be killed.

- Any procedures applied to mice that result in abnormal behaviours (e.g. lethargy, piloerection, hunched posture, reduced mobility, abnormal respiration, sunken eyes) that equate to significant harm will result in those mice being humanly killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild severity is expected for non-tumour bearing animal models that will be bred under the protocol 1. These animals may include GA Cre lines, Tracer animals (e.g. expressing GFP to label particular cell populations), wild type and immune compromised mice. We expect that animals this severity to affect

>99% of the non-disease animals in protocol 1 (total of 1000 mice).

- Moderate severity is expected for cancer animal models. We expect that under the breeding protocol 2 only <50% (<750 mice) of animals will experience this severity under this protocol since most animals may be transferred before tumour onset and thus will be considered of mild severity. Under experimental protocol 3, we expect that 80% of animals will experience moderate severity associated with spontaneous tumour development and progression (800 mice). Because most animals will be transferred to the protocol before tumour onset, we expect that ~20% (200 mice) will either not

develop tumours or culled before tumour onset and thus will be considered of mild severity. Under experimental protocol 4, we expect that 80% of animals will develop tumours after implantation and will experience moderate severity (4000 mice), we estimate that with the remaining animals (1000 mice) may not develop tumours and will experience only mild severity.

In summary, for this license we expect that 2950 animals will experience mild severity while 5550 animals will experience moderate severity associated with tumour growth.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our main goal is to understand how tumours progress, disseminate and evolve to aid the development of much needed therapeutic strategies to treat cancer patients. Most of our work focused on basic understanding of cancer cells and for that we have used and will continue to use suitable in vitro systems to address these questions. However, cancer is a very complex disease, and in particular the complexity of the interactions between cancer cells and their microenvironment is very difficult to reproduce in vitro, as well as the process of cancer dissemination and evolution. To do so, we will need to use systems that closely match the complexity of human cancer, and in particular that have the following criteria:

- Tumour microenvironment, preferentially matching the tissue of origin of the cancer, composed of other cells that surround the tumours, such as fibroblasts, blood vessels, immune cells;

- Blood supply to enable cancer cells to migrate to distant sites and form metastasis in other organs and that can be used to drug delivery;

- There are no systems in the laboratory that can achieve these 2 criteria except a live animal. Therefore, animals are essential to our programme.

Which non-animal alternatives did you consider for use in this project?

In our laboratory we have successfully used 3-D organotypic tumour models, organoids and 3-D spheroids to study for example the mechanisms of cancer cell invasion, the first step of the metastatic cascade. These models allow cancer cells to grow in 3-D environments that partially recapitulate how they grow inside the body. More recently, we have also established 3-D heterotypic cultures of cancer cells and associated fibroblasts to

look at the interaction between cancer and one component of the tumour microenvironment. These type of model systems are very useful, can be easily established and provided significant understanding that allowed us to refine our animal experiments.

In addition, to develop non-animal alternatives for this project, we have established collaborations with collaborators to establish a 3-D based flow chamber system to study angiogenesis to identify factors important for this process prior to animal studies. These simplified systems are advantageous as they

allowed us to screen different molecules in a very fast and effective manner to refine our animal experiments and therefore allow us to use less animals.

Why were they not suitable?

The laboratory systems we have established are very valuable and can address discrete defined questions and generate valuable knowledge. However, they will never fully recapitulate the complexity of a live tissue that contains blood vessels, lymphatics and cells of the innate and adaptive immune system. All of which work in an integrated manner to facilitate tumour growth and spread. For example:

- In our 3-D systems we may be able to enquire how specific factors impact angiogenesis but we will not be able to address how changes in angiogenesis impact the surrounding environment, e.g. immune infiltrates, fibroblasts. This integrated knowledge is key for us to understand how the different cellular compartments that constitute a tumour interact and impact tumourigenesis.
- Additionally, the metastatic cascade is very difficult to model in vitro as it involves several key steps: local invasion, intravasation/extravasation to and from the blood vessels, surviving in the circulation, ability to attach to other organs and grow to form metastatic lesions. While some steps can be modelled using 3-D in vitro systems, the complexity of the entire process cannot, in particular the colonisation of distant organs and metastasis formation is very difficult to reproduce in vitro.

Thus, although in isolation these in vitro systems are advantageous, they lack complexity and cross talk between the different components that co-exist in an in vivo tissue.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers present the maximum estimation mouse usage based on our current and proposed programme of work.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

- 20 years of experience in designing, running and publishing research articles means I am well prepared for training and supervising my staff in effective ways of designing and executing a project. I believe this experience is highly valuable when designing in vivo experiments. In addition, we recently started using orthotropic mouse models for breast cancer for which we acquired invaluable knowledge to help designing our project and refining our experiments.

- In preparation for this license, I have become familiar with the excellent NC3Rs EDA tools. I will be introducing these tools to all of my team with the instruction they should become familiar and where valuable, to use them in their experimental designs. The NC3R EDA tools can improve experimental design and increase my staff's knowledge and understanding of in vivo studies. I have also acquired the necessary training prior to writing this license, which helped also with the design of effective in vivo experiments while always considered ways to implement the 3Rs.

- In the last years we become familiarised and have used successfully the on-line power calculation tool (http://biomath.info/power/). This has been instrumental to design animal experiments that have been peer reviewed in our recent grant applications. Our resulting experiments have also produced data that provide statistical significance and thus more scientific value.

- In our laboratory, we always start in vivo work with a clear hypothesis. This is key so that we can design an experiment with defined goals, sufficient numbers of mice, a clear monitoring strategy, a pre- planned means of analysis, a randomisation plan and a data blinding plan. These should be clear and agreed before the experiment commence.

- We will take advantage of current literature, our in vitro work, for example using 3-D organotypic systems, refine our questions to minimise the use of animals. For example, if a therapeutic change is desired, we would first conduct multiple in vitro studies including, if possible, 3-D multicellular pathology-mimicking models to prove that indeed, there is a potentially useful biological effect. Only then would in vivo experiments be considered. Sometimes the justification may come from studies from other laboratories that we will then incorporate in our study. The level of effect helps to determine the expected % biological change that will inform the power calculations.

- We use multiple imaging tools to monitor tumour progression, metastasis and response to treatment. This allows us to follow temporally the change in tumour growth and

activity during an experiment without the need to kill the animal. These tools include bioluminescence, MRI and radio-imaging (SPECT/CT and PET/CT).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Where the scientific question requires modification of gene expression in a target tissue, whenever possible we will always utilise GA mice designed such that there is a minimum number of crosses with other mice to achieve the desired genotype. The wastage in animal life to achieve effective genotypes will always be considered.

- Pilot studies are key to minimise the use of mice in our studies as this will allow us to know with a high degree of confidence the correct number cells to form tumours, the time frame for tumours to grow, whether the tumours spread, the correct dose of drug to achieve the maximum biological effect, the correct dosing to achieve optimal pharmacokinetics. When these facts are in place we can accurately design good experiments to address our questions.

- We are keen to make available spare tissues from our studies to others to use as a means of performing either pilot or even experiments, without the need to use additional mice. For example, we have recently provided control mouse mammary tumour sections to colleagues to test whether this could be a good model to investigate the formation of tertiary lymphoid structures without the need to using additional animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically Modified Mice (GEMM). Some of these mice will naturally develop cancers of interest (including pancreas and breast) so that we can investigate the biology of the developing disease and test our treatments in a model that most closely matches human diseases. For most models, the mice will not develop more than mild pain or discomfort during early stages of tumour development.

However, cancer is a progressive disease and thus, all animal models will be monitored closely to ensure their well fare. Tumour size will be monitored to ensure that the total

tumour burden of SC/mammary tumours do not exceed 1250mm3 and internal tumours (pancreatic) will not exceed 800mm3. MRI monitoring of deep tissue tumours (e.g. pancreatic tumours) provides confidence we can deliver this intention. Some of the GEMM we will use are not tumour models but will be used to label specific tissue compartments and components of the microenvironment (e.g. blood vessels).

Mice bearing transplanted tumour cells/tissues. Cells or tissues derived from mouse or human tumours or circulating tumour cells will be injected or implanted into mice subcutaneously (SC), orthotopically or intravenously (IV), as required by the scientific question. Experienced handlers will inject the SC and IV cells/tissues minimising pain and discomfort to mice. Mice will require anaesthesia and surgery for orthotopic injections and implantations of organotypic gels and will receive peri-op analgesia to minimise discomfort. Surgeries will be conducted by highly trained staff. When possible, orthotopic injections to the pancreas will be conducted using an echo-guided approach that is less invasive than a surgery, to minimise pain and discomfort. In some cases, genetically manipulated cells that contain bioluminescence or fluorescence will be used to accurately monitor tumour development and response to therapy using non-invasive methods, thus minimising pain and harm to animals.

All experimental mice are routinely examined for their health status and any behaviours or appearances that suggest pain or discomfort will be monitored closely with the local BSU staff and as required, the NVS.

Why can’t you use animals that are less sentient?

Most of our experiments require that tumours will develop over a time period. This is because the pathologies are a result of complex interactions between cancer cells and the surrounding tissue. Moreover, it is not possible to use animals that are terminally anaesthetised. In addition, it is important to use a simple mammal in order to closely reflect the biological processes in humans that we are trying to investigate. While non-mammalian models (drosophila, xenopus) are proven very useful, they do not recapitulate the biology that occurs in humans. We have used in the past 48hrs post-fertilisation Zebrafish embryos as a model to investigate cancer cell invasion. However, while this model can recapitulate several steps of the metastatic cascade, it does not provide the time frame to understand this process. Mice are the simplest mammals that through decades of research provides us with confidence that our experimental designs are likely to be as close to our discovery and therapeutic goals as possible.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- We will use MRI and other minimally-invasive imaging methods available at out institution to improve the analysis and monitoring of metastasis and internal tumours (e.g. pancreas) that develop either through orthotopic implant or growth in transgenic mice. This will be useful for example to use mice that have similar volume tumours at

Day 1 of a protocol, instead of starting therapy on a cohort at a predetermined time interval after injection. This also allows longitudinal studies that formally required cohorts of mice to be killed at intervals, thus saving many mice. The MRI protocol has the immediate benefit that there is limited chance that an animal develops an internal tumour that exceeds home office limits. We will work closely with colleagues in our institute implementing such methods to guide our experiments.

- We will strive to use transgenic mice in which mutations are induced specifically and conditionally in the cell population of interest, using for example Cre-LoxP conditional alleles and Tet-On and Tet-Off systems to temporarily control the expression of oncogenes. These mice should not display a phenotype until the mutation in the candidate gene is induced.

- In all our experiments we will set humane endpoints and write an experimental protocol, which will include details of possible adverse effects. For example, when administering substances or cells to animals, the route used for delivery will be such as to achieve “best practice”, that is to minimise or avoid adverse effects, while minimising the number of animals used, and maximising the quality and applicability of results. For that reason, we propose in this project licence a variety of routes of administration of substances and cells to achieve the scientific objectives, while minimising the waste of animal’s lives.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We have been following the ARRIVE guidelines and also use the NC3R EDA tools on line.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institute hosts regular updates in experimental animal usage, including new developments in 3Rs that I try to attend or send representatives to from my team. We also have colleagues at our institute that attend/spoke at NC3R events with whom we can discuss the best practices on how other researchers conduct their research.

143. The use of immunological tools for monitoring farm cattle health and prevalence of bovine tuberculosis (btb)

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cattle, tuberculosis, mycobacteria, immunology

Animal types	Life stages
Cattle	juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To determine immunological base line parameters of farms that have suffered bTB outbreaks compared to animals on farms with no recent outbreaks.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A wealth of knowledge has been generated on the immunological response of cattle to Mycobacterium bovis, the mycobacteria that cause bovine tuberculosis (bTB). This knowledge has been largely acquired from animals held in tightly controlled experimental

conditions. There is now a need to determine the extent to which this knowledge can be applied for enhanced disease diagnosis and infection control in animals managed under commercial farm conditions. There is also a need to determine the extent to which different tests/protocols will be accepted by farmers and regulatory authorities and/or are practical under commercial farm conditions.

What outputs do you think you will see at the end of this project?

The aim of the experiments to be carried out under this licence is to gain a greater understanding of the bovine immune response to mycobacteria under commercial farm conditions by evaluating immune processes, tools and reagents previously generated with cattle housed under experimental conditions. The expectation is that these markers could be used to design and develop novel diagnostic and prognostic tests to maximise detection of infected animals and thus eliminate the risk of infection transmission to non-infected animals (cattle to cattle transfer). We also expect to generate novel markers of infection. Our laboratory has acquired state of the art equipment that will permit us to carry out the analysis of the immune response at different levels and we expect this to allow us to generate novel findings on the host:pathogen interaction during bTB infection.

Any new findings will be published in international, peer-reviewed journals and presented at national and international meetings and conference. Due to the fact that we will be approaching farmers to bleed animals on their farms we also expect to communicate to farmers, community-based farming groups and field veterinarians on the progress of our studies.

Where appropriate, we will seek to protect intellectual property through the submission of patents.

Who or what will benefit from these outputs, and how?

The control of bTB in cattle poses a significant financial burden due to test and compensation costs on the GB taxpayer. In 2017, over 40,000 cattle were slaughtered, which cost the GB public purse over £100M (references). Further, there are direct costs to the farmer such as, loss of product, additional husbandry, etc. due to a herd being under movement restrictions until it regains its bTB-free status. bTB is a progressive chronic inflammatory infectious disease with the potential to directly impact on animal health and welfare. It is also a zoonotic disease and poses a risk to human health. Control of bTB could have benefits in reducing the prevalence, incidence and spread of bTB in the cattle population and could also reduce the severity of a herd breakdown regardless of whether infection is introduced by wildlife or cattle. Ultimately, the control of bTB would result in increased animal health with a concomitant increased in productivity and a reduction in the costs of the control of the disease to the taxpayer and farmers. Control of bTB will also result in a reduction on the risk of zoonotic TB in humans, which was one of the major drivers for the introduction of bTB control measures in the 1900s. It is also relevant to state

that any lessons learnt of the control bTB will inform developments in the field of human TB.

The risk of a herd suffering bovine tuberculosis outbreaks is positively correlated with its size. Herds with over 300 cattle and which have suffered prior bovine tuberculosis outbreaks have been identified as most likely to suffer new outbreaks (chrome-extension://efaidnbmnnnibpcajpcglclefindmkaj/viewer.html?pdfurl=https%3A%2F%2Fassets.publishing.service.gov.uk%2Fgovernment%2Fuploads%2Fsystem%2 Fuploads%2Fattachment_data%2Ffile%2F923195%2Ftb-epidemiology-england- 2019.pdf&clen=2856273&chunk=true). Thus, it is likely that the farms we will work with will house a large number of cattle. In order to maintain a balance between the number of animals per farm to remain representative of that farm and comparison between potentially different management systems, we propose that the minimum number of farms to be sampled will be four and that the maximum number of animals per farm to be sampled will be 150. This allows the sampling of a representative number of animals on farms that use different management systems.

The development of improved tests for the detection of animals infected with M. bovis requires an improved understanding of the immune response against mycobacteria. Infection with M. bovis is a chronic infection that is known to evolve with time; it has also been shown that the immune response to mycobacteria is affected by the application of the skin test (https://www.sciencedirect.com/science/article/pii/S0165242704001989?via%3Dihub). Evaluation of responses after application of the skin test may yield results that would allow the development of diagnostic targets different to those detected by current tests. Therefore, we seek to bleed animal for up to six times per year to allow for monitoring of the immune response over time before and after application of the statutory skin test. Thus, each animal will be bled for a maximum of up to 30 times during the period of the licence.

On a broader aspect, it would be expected that results from the experiments carried out under this licence will benefit stakeholders in other countries also suffering from bTB in their herds.

We also expect that one major output of these experiment will be scientific knowledge on the nature of the immune response of cattle on commercial farms. It is worth mentioning that the knowledge that we expect to generate is likely to have a wider application in our understanding of the bovine immune response to a range of diseases other than just bTB, and to micro-organisms other than mycobacteria. This knowledge in turn is likely to generate patentable intellectual property.

How will you look to maximise the outputs of this work?

We intend to collaborate with colleagues at national and international level. As mentioned above, we will seek to publish our data in international, peer-reviewed journals and

presentations at national and international meetings and conferences. There is currently the possibility of publishing different approaches, whether successful or unsuccessful in pre-print portals, such as bioRχiv, which makes the information available to the international community whilst allowing the community to comment on the publication.

Where appropriate, we will seek to protect intellectual property through the filing of patents, which in turn will allow the publication of data in peer-reviewed journals.

Where appropriate, we will seek to engage with companies to exploit new findings with the aim of reducing the burden of bTB in the UK and other parts of the world.

Species and numbers of animals expected to be used

- Cattle: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Bovine Tuberculosis (bTB) is a disease of cattle and there are currently no in vitro or in silico models that would permit the study of the host pathogen interaction in this disease. The best model for the study of bTB are cattle, which are the target species affected by this disease.

In this project we will be studying animals directly in commercial farm settings, which will allow an interpretation of data that reflects the reality of how this infection affects animals on farm through the course of time.

Typically, what will be done to an animal used in your project?

Animals will only experience the procedure of drawing blood either via the tail vein or jugular vein dependent on volume collected. For small volumes blood will be sampled from the tail and for large volumes bleeding from the jugular vein; the latter will occur only when there are appropriate resources to contain the animal for safe bleeding. In the case of young animals (< 4 months) a halter and two operators may be sufficient. For animals older than 4 months, there will be a need for animals to be contained in a crush

What are the expected impacts and/or adverse effects for the animals during your project?

Theoretically, there may be some haematoma development after bleeding . We will seek to prevent/control haematoma formation by applying pressure on the site immediately on removal of the needle. From prior experience, no haematoma/thrombosis formation has been observed/recorded after this procedure has been performed.

When bleeding from the tail vessels, it is possible that the site may be infected; to avoid this, the area of the bleeding will be cleaned prior to sampling.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mild

What will happen to animals at the end of this project?

- Set free

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Currently there are not ex vivo nor in silico models for the study of the bovine immune response to mycobacteria. The most relevant model for the study of the BTB is the target species, which in this case is cattle.

Which non-animal alternatives did you consider for use in this project?

The validation of laboratory findings in farms animals requires by necessity live animals and there are not any non-animal alternatives.

Why were they not suitable?

The purpose of this project is to validate laboratory findings in field settings with a view to develop novel diagnostic tests. This needs to be done in live animals of the target species in which these tests will be used; therefore, no in vitro surrogates can be used.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We expect to be able to sample between 5 and 10 dairy and beef farms per year. The average number of animals per dairy herd is of approximately 95 animals, whilst the average number of animals per beef herd is 23 animals. Sampling an equal number of beef and dairy farms would provide an average of 75 animals per farm. We estimate that we will be able to recruit between 5 and 10 farms. It is our expectation to be able to sample these farms between two and three times per year. Thus, we have calculated an average number of 75 animals per farm. Although the aim of the project is to recruit between 5 and 10 farms, for the purposes of this calculation we have used the average of the number of animals in 8 farms, which gives a rounded up value of 600. The increase in the number of animals is requested to ensure that we will be able to sample a minimum number of establishments, which will allow for the potential comparison of different management systems. It is important to bear in mind that the numbers indicated above are averages and that dairy farms in which bovine tuberculosis can occur are generally farms with large numbers of animals. We propose that we will sample a maximum of 150 animals on up to four establishments. This allows the sampling of a representative number of animals on farms that use different management systems.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To our knowledge, an experiment of this nature, i.e. to evaluate the immune response to mycobacteria in cattle on farm has not been carried out and therefore there is currently no data on which we could base our calculations.

Nevertheless, we have sought to sample as representative a cattle population of working farms as possible, hence the idea of sampling from both beef and dairy farms. BTB is a chronic disease which evolves through time, accordingly, there is a need to bleed the same animals (if available) for several times per year. We anticipate that the average number of samples taken from each animal will be three per year, although there may be a need to sample individual animals up to six times per year. The increased number of samples may occur after statutory testing of animals for bovine TB to allow us to assess the effect of the application of the skin test on the immune response to mycobacteria over time. Effects of the skin test on the immune response to mycobacteria could be used to determine prior exposure to M. bovis and therefore be used as potential diagnostic markers.

We believe that bleeds, as currently proposed in the license will be sufficient to address the scientific need of the project.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As mentioned above, there is currently no experimental data upon which we can base our calculation to optimise the number of animals we will use and this will be dependent on the

results we obtain in our experiments as well as the permissions we are granted by the farmer in terms of access to animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Cattle are the target species of bTB and therefore the appropriate species to be used to achieve the objectives outlined in this license. Bleeding will be performed using standard venepuncture methods for the amount of blood we are seeking to obtain from each animal to allow us to carry out the research we have described in this proposal using state of the art technology. We will work with farmers and veterinarians to minimise pain, suffering and distress or lasting harm to the animal.

Why can't you use animals that are less sentient?

Cattle are the target species of bTB and are therefore the only appropriate model in which to study this disease. It is a chronic disease for which animals are required to live over periods of months and preferably years, which is the reason why this experiment is being proposed over a period of five years.

Cattle on commercial farms are the target for the evaluation of novel diagnostics tests previously developed in animals kept in tightly controlled experimental environments. There is not any other animal that can be used for this purpose.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will be in frequent communication with the farmer and the veterinarians advising the farm to try to minimise any harm to the animals every time they are bled. We also intend to coordinate our sampling with other routine sampling times to minimise the number of times the animals require handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. We will continue to follow the scientific literature to refine our

experimental approaches with a view to maximising the information we obtain from the blood we withdraw from animals; in time, this will allow a reduction in the amount of blood necessary to obtain similar observations and/or reduce the necessity of extra bleeds. By attending AWERB meetings and Home Office refresher courses on animal handling we expect to keep up to date with the literature that will allow us to perform our experiments in the most refined way possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will be attending the AWERB as and when required and I shall also attend refresher courses as and when required. I will follow advice from our HO liaison officer and the NIO on the documentation and courses to attend.

144. Production, breeding & cryopreservation of genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words
Genetically altered mice, Genes, Breeding, Biomedical research

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to generate, breed and maintain genetically altered mice and supply them for research into the study of normal and abnormal physiology, development, biology or behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The functions of many genes and their products, either individually or in ways they interact to produce their intended effects, or how they are dysfunctional in disease is currently unknown or not fully understood. To understand better the impact of genes on normal physiological and abnormal disease processes requires, when necessary and justified, the use of genetically altered animals.

What outputs do you think you will see at the end of this project?

The investigations carried out on animals bred under this licence hope to obtain new knowledge with respect to basic mechanisms of physiology, behaviour, development and cell biology. Any animals bred under this licence which will be used on other Projects will also have publications generated from the research. GA animals may be culled for invitro work or tissue analysis to provide information that is used to contribute to current or future in vivo projects.

Who or what will benefit from these outputs, and how?

Short term benefits

The short-term benefits from this work include the development of basic understandings of how genetic modifications affect biomedical processes.

Long term benefits

We hope that any new information and understandings found in the work carried out under this licence will lead to new therapies and treatments.

How will you look to maximise the outputs of this work?

New information and data from these investigations will be shared to the scientific community in peer- reviewed scientific journals, meetings and seminars. Some end-users take part in sharing knowledge to the wider community through public engagement with relevant charities.

Species and numbers of animals expected to be used

- Mice: 21500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are the most commonly used animals in research due to their similarity to man and are in widespread use in biomedical research to study functions of a mammalian system for translational studies; they are also the species with the lowest sentience that can be used to meet the scientific objectives of this project and projects of the end user.

Adult mice are needed as they are sexually mature and able to provide the offspring needed for study.

The studying of the development of diseases or biological processes may require animals to be used throughout their life span, we do not intend to use animals beyond 1 year old.

Typically, what will be done to an animal used in your project?

The majority of GA mice will undergo the removal of ear tissue (biopsy) for genotyping purposes and then for tissue collection post mortem, some of these may need a secondary biopsy for confirmation of genotype. Some of these mice with be used in other approved project licences under 'continued use'.

Mice used for breeding will not be bred before they reach sexual maturity and the majority will cease breeding at 6 months old.

Some mice will be administered substances to induce genetic alterations such as intraperitoneal injections of tamoxifen.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals (around 90%) are not expected to have negative phenotypes due to their genetic alteration/s but most will experience momentary pain during biopsy. Those which undergo treatment to induce genetic alterations via the administration or application of a substance may experience mild pain and possible weight loss.

Some end-users may require the use of animals that have moderate phenotypes (e.g. tumour growths and hydrocephalus), these may experience moderate level of pain suffering and lasting harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect most animals to experience a sub-threshold level of severity. Animals which undergo a second ear biopsy will experience a mild severity (around 5% of total animals).

Mice who receive treatment to induce genetic alterations (e.g. tamoxifen, doxycycline) are also expected to only reach a mild severity (no more than 10% of total animals based on current usage).

Mice with adverse phenotypes are not expected to be over 10% of the total number of animals used.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The mouse is the most commonly used animal to study the effects of genetic alterations. Mice often have high fecundity and are reliable breeders. Users of the mice provided often need to study phenomena, diseases and biological processes in a whole animal and tissues taken from mice.

Which non-animal alternatives did you consider for use in this project?

End-users often use well established cell lines for in vitro work. Applications for the use of a new genetically altered strains to the facility (i.e. already established strains but new to the facility) must be approved by the AWERB sub-committee comprising of NVS, NACWO, BSU Manager, a Project Licence holder and a Personal Licence holder to help ensure that alternatives have been considered.

Why were they not suitable?

In vitro and in silico models cannot completely simulate the complexity of a living mammalian system which will be under study using the animals in this licence.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals expected to be used under this licence has been estimated on past usage and planned work in the near future.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

A user's requirement of the animals must be confirmed before the animals are introduced and bred in the facility. Cryopreservation, strain sharing and/or repurchasing will be encouraged rather than maintaining lines which are not being actively used.

Unnecessary production or importation of genetically altered animals will be avoided by searching resources such as animal suppliers, biomedical research institutions and industry forums.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where possible the knowledge of which GA strains are held in the facility is shared to all users, this helps to promote sharing of tissue and collaborative work. The facility has over 20 years experience of breeding GA mice. The staff in charge of breeding and maintenance of all GA lines (in accordance with the 3Rs) and are in regular contact with users to continually assess and adjust breeding to provide the numbers required for their research without over breeding.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are using genetically altered mice for this project. The majority of tissue taken for genotyping will be taken from the ear which provides reliable results and also acts as an identifiable marker. Transgene inducing or deleting agents may be administered by injection (e.g. subcutaneous and intraperitoneal), in diet or drinking water (with the exception of tamoxifen) or the implantation of a slow release pellet subcutaneously on one occasion. The mice produced in Protocol 1 are not expected to have any adverse phenotypes.

Why can’t you use animals that are less sentient?

The knowledge sought by our end-users require the studying of processes in a mammalian systems, by using mice we have chosen a model that has the lowest sentience.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The facility has many years' knowledge of breeding and maintaining genetically altered animal. Any mice that are expected to have potentially adverse phenotypes will be monitored using a general health monitoring sheet.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We shall follow Laboratory Animal Science Association good practice guidelines for the administration of substances and the Efficient Breeding of Genetically Altered Animals framework published by the Home Office.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I attend NC3Rs, LASA and IAT webinars and conferences regularly to ensure I am aware of advance in the 3Rs and the facility has an experienced NVS and NACWO who also have up-to-date knowledge of any advances. I am also part of industry forums which provides a place to find out up-to-date information and vital contacts to help improved the 3Rs.

145. Discovery of cancer genes and assessment of their therapeutic potential

Project duration

5 years 0 months

Project purpose

Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer gene, Therapy, Cancer immunology

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our goal is to identify genes which cause or contribute to the cancers and their spread.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

In the UK 350,000 new cases of cancer are diagnosed each year and there are around 165,000 cancer- related deaths. If we are to reduce cancer incidence and the associated suffering and deaths, we need to identify tumours earlier and develop new therapies.

Cancer cells have alterations in their DNA, so called genetic changes. These genetic changes allow the body's immune system to detect and kill cancerous cells which can keep most of us cancer free for most of our lives. In many cases however, cancers can make them selves invisible to the immune system and these can then grow unchecked. To better control cancer we need to understand how they hide from our immune system.

What outputs do you think you will see at the end of this project?

New medicines, namely drugs that offer potential new treatments for cancer.

Basic Science benefits:

Information on genes involved in cancer which may be useful for diagnosis

New information that might enable new cancer drugs to be developed

Publications and presentations at meetings

Genetically engineered mice that can be used by others in the scientific community.

Who or what will benefit from these outputs, and how?

Society will benefit from knowledge generated as this provides better understanding of diseases, disease mechanisms and methods to treat and/or prevent them. Scientific knowledge is gained over the shorter-term (2-5 years) while drug discovery and development will take 10-15 years.

Research institutes, pharmaceutical companies and non-governmental organisations will be able to take information produced by our studies and use it to support basic research and the development of drugs that ultimately provide benefit to patients.

How will you look to maximise the outputs of this work?

We will collaborate with other experts, combined efforts will increase output.

We will publish scientific papers in open access journals and deposit papers in appropriate archives thereby disseminating knowledge.

We will share both positive as well as negative results with the scientific community so that others are aware of both successful and unsuccessful approaches.

We will file patents, thereby placing in the public domain detailed knowledge of the discoveries we have made.

We will use the ARRIVE guidlines when publishing our work.

Species and numbers of animals expected to be used

- Mice: 14,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse was selected for this project because of the availability of advanced technologies to genetically alter mice. This is done by adding or removing genetic information at very early embryonic stages, before an embryo has implanted in its mother's womb. The methods to do this are very efficient so fewer animals are needed compared to achieving the same goals in another species.

Adult mice develop many forms of cancer that are similar to cancers in humans, for instance lymphomas, brain, colon, ovarian and prostate cancers. Just like in humans cancers develop in older mice more often than younger ones. Genes have been identified which when damaged cause cancer in humans and mice. One example is a gene known as p53. Mice and humans that have just one copy of the p53 gene instead of the normal two copies, develop cancer much earlier than normal.

Many strains of mice are genetically identical. This enables tumours to be caused by injecting them with cancer cells. This technique has allowed experiments to be performed to discover anti-cancer drugs. Indeed almost all cancer treatments available today have been tested for their ability to cure tumours in mice. Mice, when used in this type of work, give valuable insight into basic disease mechanisms as well as providing a means to test new therapeutics.

Typically, what will be done to an animal used in your project?

To produce transgenic mice fertilised embryos are collected which are manipulated under the microscope. These are transferred to foster mothers who become pregnant, give birth and rear their pups.

The embryo transfer is done under general anaesthesia so the animal will be unconscious and unable to feel pain. A small cut is made in the skin to place the embryos in the reproductive organs (uterus or oviducts). Typically, the mice recover very quickly from surgery, they are actively running around, eating and drinking after 1 hour and are fully recovered within a few days.

A small number of male mice are vasectomised, which are used to mate with foster females. Vasectomy is conducted on anaesthetised males by making a small scrotal incision and cutting the thin tube carrying the sperm. The incision is closed and the mice recover from the anaesthetic within 60 minutes or so. A week or two later the wound will be healed and the mice can be used for mating with females.

Transgenic mice in this project will be bred and weaned. Where the line is not pure bred a tiny piece of tissue from the ear is taken to identify the mice and the bit of tissue that is removed used to identify the genetic alterations they carry.

Transgenic and normal mice are used to study cancer. Cancers may arise spontaneously in some strains but in others we induce cancer by injecting them with cancer cells or with viruses that carry genes which cause cancer. In some cases tumour cells are placed just under the skin of the mice. The growth of these cancers is monitored by feeling the small tumour lump under the skin and the mice are humanely killed before the cancer grows very large (less than 1.2cm2).

In other cases we need to precisely control where the cancer develops, for instance to induce prostate cancer we inject the prostate gland under general anaesthesia. In such a situation the mouse is unconscious and unable to feel pain from the small incision and injection. Typically the mice recover very quickly from these surgeries. Over the following weeks or months they are monitored closely for tumour development. As the prostate can not be seen, we monitor tumour growth by imaging - very much like a scan in a hospital. To do this we inject a dye into the body cavity and while the mouse is anaesthetised we perform a scan to see if a cancer is growing or has spread. We do this monthly to identify tumours before they become too large and impact the health of the mouse. Once a tumour reaches a certain size the mouse will be humanely killed and the tumour recovered for analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the animals in this project should not experience any adverse impact as they are used for mild procedures like breeding. Mice are typically breeding for 4-9 months.

Some of the animals will experience transient pain from the minor surgery required for embryo transfer, vasectomy and inducing tumours in specific organs. These procedures will be conducted under anaesthesia so the animal will remain in a state of sleep/unconsciousness during the surgery. Once they have recovered from anaesthesia any pain will be managed with drugs for a few days.

The procedures used for tumour cell inoculation under the skin will involve needle pricks. In procedures which involve anaesthesia, the mice will be expected to be subdued and lethargic for an hour or two but then recover fully as the affects of the injection and/or anaesthesia wear off. Some animals may have an altered immune system that may make them more susceptible to infection. To keep mice with immune deficiencies healthy, they are maintained in a very clean facility where they are not exposed to harmful viruses or bacteria.

The tumours will be allowed to grow to a size where they may cause some discomfort and may cause other clinical signs. We will carefully monitor mice with tumours to make sure

that the tumour is not having a major impact on the health of the mice. We monitor weight, breathing, mobility and general condition of the mice. We also routinely measure tumour size, which may involve feeling it through the skin or using an imaging technique like ultrasound.

Tumour growth will occur over a period of between two months and one and a half years and can be unpredictable, so monitoring will be continuous. Tumour bearing mice will be humanely killed at the point that we judge that the tumour burden is having a significantly health impact or if tumours don't grow within 18 months.

All animals will be killed humanely at the end of the procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mouse : Mild severity : Greater than 96%

Mouse : Moderate severity: less than 4%

What will happen to animals at the end of this project?

Killed
Kept alive
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancers are usually composed of tumour cells mixed with and normal cells. As they grow new blood vessels form to provide the tumours with oxygen and nutrients. Very often tumours are attacked by cells from the immune system which recognize the cancer cells as "foreign" and in some cases the tumour is controlled or even eliminated. In many other cases the tumour is able to "hide" from the immune system.

When we detect cancers they have usually been growing over a very long period of time, years in humans and many months in mice. During this time the cancers change so they are composed of a mixture of similar but non-identical cells. Cancers that arise in different people have some similarities, but each one is unique.

To control cancer we need to identify the genes which are able to support the growth of cancer and the mechanisms cancers use to hide from the immune system. Studies of

cancers isolated from people and mice over the last 10 to 15 years have given us long lists of "cancer genes". To identify which combinations of genes are contributing to the cancer's initiation and growth requires studies in animals because cancer cells grown in the laboratory are in a very simple environment compared to when they arise naturally in humans. It is not possible to replicate the complexity of how a cancer grows, interacts with normal cells and manages to evade the immune system in a laboratory setting.

Which non-animal alternatives did you consider for use in this project?

Cells isolated from some cancers can be grown in the laboratory. These culture systems have improved in recent years allowing cancer cells to grow into tiny but more complex structures that better resemble some aspects of normal tumour growth. These so called "organoid cultures" are three- dimensional cultures usually composed of a small number of related cell types. While their three- dimensional nature can more closely resemble some aspects of simple tissues they are very simple compared to the highly complex environment one finds in a mouse or human tumour. Organoid cultures are useful for conducting screens for drugs or mutations that can alter a cancer cell's growth but these systems to not have an immune system component, thus drugs which prevent a tumour hiding from the immune system can not be found using such simple systems.

Why were they not suitable?

To study how a cancer evades the immune system and to find rare immune cells which can kill tumour cells it is necessary to study cancers in the presence of a functional immune system. This can't be replicated in the laboratory. Similarly, the process whereby a cancer spreads from its primary site can only be studied in the whole animal system.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals estimated to be used for this project are based on many years of experience in generating mice with altered genes. They are also consistent with the usage in the existing project license.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Genetic manipulation: More than 90% of the animals used in this project will be used for establishing and breeding combinations genetic alterations together. The numbers of mice

can be estimated from prior experience using genetically modified ES cells to make mice as well as the probability of generating mice with the desired combination of alterations based on patterns of Mendelian segregation.

Where possible we establish mice which breed true.

We test the performance of each genetic alteration before breeding them together.

Cancer studies: Around 5-10% mice in this project will be used to deliver the core scientific objectives, which involve inducing cancers and collecting the resultant tumours and analysing them.

To reduce mouse numbers we will:

Conduct careful quality control on all samples used, such as cell lines and viruses, to make sure that when they are injected into mice there is a very high chance that useful data will be collected.

Use standard operating procedures to ensure that we get the maximum amount of useful biological information from each mouse.

Consult widely to identify and where appropriate introduce improvements in methods to maximise the recovery of data from every mouse

Apply technologies which enables us to gain the maximum amount of information from every sample collected.

Use the NC3Rs Experimental Design Assistant

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

There are many measures we take to reduce mouse numbers:

We use colony management software to keep track of mice in the animal room, set up just the right number of breeding pairs to produce the mice we need.

We conduct pilot experiments using small numbers of mice to assess the performance of a cell line or virus before initiating larger experiments, or not conducting them at all in cases where the pilot does not provide a good basis for continuing.

We stop breeding and preserve mouse lines in cryogenic storage if they are no longer required

We use the most advanced and efficient methods to make genetic mutants which avoids cycles of mouse breeding.

We share post-mortem tissues

We limit "noise" by controlling as many variables as possible including genetics (inbred lines), age, sex, and environment.

In induced tumour models we use randomisation and blinding to control for operator bias.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice with cancer pre-disposition mutations

We use mice with genetic alterations (mutations) in cancer causing genes. If these mice are available from other investigators or public repositories we will import these. If they are not available we'll generate these ourselves. We use refined models so that cancer is limited to the organ we are studying - for instance the prostate.

Some genetically altered mice develop cancers spontaneously. To limit their suffering, we conduct regular screens for signs of cancer and if detected more frequent screens are then implemented.

Mice with immune deficiencies

We use mice with immune-deficiencies to explore the role of aspects of the immune system in controlling cancer. Although, some parts of the immune system are missing in these mice, they are very healthy in the environment in which they are kept.

Transplantable cancers

To conduct controlled experiments, we use cancer cell lines which we implant into mice, either normal mice or ones with immune defects. The tumours are usually injected under the skin which allows us to monitor the size of the tumour to limit any suffering.

Organ specific cancers

These are induced by combining a specific genetic background which may pre-dispose the mouse to develop a specific cancer type with local induction of the tumour by administering a cancer causing agent like a virus. In such cases we will endeavour to design the experiment so that the growth of the cancer can be detected by an imaging technique which will allow us to intervene to prevent suffering.

Why can’t you use animals that are less sentient?

Cancer is a late onset disease in adults. It develops from a single cell that arises in mature organs over many months, thus an immature life stage is not suitable. The immune system is also not mature at an earlier stage (immature life stage).

Some aspects of cancer can be modelled in zebrafish, for example they develop a type of skin cancer known as melanoma, but zebrafish have a very different immune systems to humans and mice, their organ systems are very different to ours and lower organisms can't be genetically engineered to the extent possible with mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

I don't anticipate many refinements to transgenic methods as these are already very standardised and efficient, though where these are reported we will test and implement them.

Where possible we will employ highly experienced animal care staff who are familiar with the specific strains, experimental activities and careful handling of the mice. Familiarity with the balance between welfare and experimental needs, observing recording and reporting expected and unexpected outcomes at the cage-side with score sheets is another essential aspect of minimizing welfare costs. An important balance needs to be struck between to much disturbance of the mice while allowing time pre- and post-procedures for acclimatisation and recovery. To improve post-operative recovery heat mats are used to warm mice as well as changes to the environment such as deeper bedding and nests can be provided.

Where possible the mice will not be disturbed unless required for routine husbandry, daily checks or experimental purposes. Environmental enrichments like tubes and nests will be provided. We will however monitor harms via observation and body weight if appropriate and in situations where this does not induce more stress to the mice.

Where possible mice will be acclimatised, for instance when moved between facilities or animal rooms before any experimental procedures are conducted.

We consult existing literature to ensure we use the latest refinements in experiments. Work will be carried out in state-of-the art facilities by highly trained technicians and scientists, all of whom are dedicated to the highest standards of animal welfare. We use imaging and/or calipers to accurately assess the size of a tumour and these are monitored against previous data to enable prediction of the possible disease course.

The scientists and technicians work closely with the trained and highly experienced personnel in the facility and the veterinary surgeon to ensure that animals experience minimal adverse effects.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I use guidance from the following sources:

The National Centre for the Replacement Refinement and Reduction of animals in Research (NC3Rs) https://www.nc3rs.org.uk

The International Mouse Phenotyping Consortium (IMPC) https://www.mousephenotype.org.

The International Society of Transgenic Technologies (ISTT).

Some of my team members are members of The Laboratory Animal Science Association (LASA) and attend an annual conference where information on best practice is often exchanged.

The PREPARE guidelines have been consulted

The Workman et al., publication, "Guidelines for welfare and use of animals in cancer research" (2010) has been consulted.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I periodically check website for The National Centre for the Replacement Refinement and Reduction of animals in Research (NC3Rs) https://www.nc3rs.org.uk which has many excellent standard operating procedures and videos and consult Norecopa, https://norecopa.no.

I check the website for the International Society of Transgenic Technologies (ISTT) for advances in the field and observations and experience in implementing these.

Implementation of a technical variation will usually be conducted with a pilot experiment to gain confidence in the actual method and its reported advantages, ideally with suitable controls. Once this has been assessed and shown to be an improvement then this will be introduced in the standard operating procedure and then implemented as a routine.

146. Molecular basis of neurodevelopmental disorders

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

autism, neurodevelopmental disorder, protein synthesis, fragile X syndrome, Syngap

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo
Rats	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to determine how molecular changes in neurons contribute to autism and intellectual disability, and to identify new treatments.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The worldwide prevalence of autism and intellectual disability is approximately 1%. Affected individuals and family members experience undue burden emotionally and financially, and institutional support is often not sufficient to alleviate these concerns. Treatment options are limited to compounds that target the behavioral symptoms associated with autism and ID, but there are no treatments that target the underlying cause. Identifying new treatments that target the core disruptions is therefore an important unmet need.

What outputs do you think you will see at the end of this project?

The outputs from this project will include new information about the disease mechanisms of autism and intellectual disability. They will also include the outcomes of testing new therapies in mouse models of autism and intellectual disabilities, which will inform further clinical research. This information will be published in major journals, presented at scientific conferences, and disseminated to the lay community through public engagement activities.

Who or what will benefit from these outputs, and how?

The major benefit of this research is the identification of new drug treatments for patients with autism and intellectual disability, which will benefit affected individuals and family members. This research will also benefit researchers in the neuroscience community by identifying mechanistic connections between molecular changes and brain function.

How will you look to maximise the outputs of this work?

Outputs of this work will be published in major journals, presented in international conferences, and shared with collaborators at all stages of development. Information will be shared whether it supports or disproves the original hypothesis, so that the data can be used as a basis for further research.

Species and numbers of animals expected to be used

- Mice: 30000
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

A thorough understanding of how neuronal function is altered in autism and intellectual disability requires investigation of an intact mammalian brain. Testing of rodent models of neurodevelopmental disorders at multiple developmental ages is key to uncovering molecular disruptions in the brain that can be targeted with new treatments.

Typically, what will be done to an animal used in your project?

We will breed animals with disruptions in genes that have been linked to autism and intellectual disability, such as the Fmr1-/y mouse model. - In some cases animals may be given drugs to study the effects on brain function

In some cases animals may be given drugs to alter gene expression

Animals may be tested for performance on learning and memory tasks

Animals may be tested for audiogenic seizures

Animals will be killed by humane methods and their brain tissues used after their death to study changes of interest

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of animals used will carry genetic mutations with no associated harmful phenotype. A small number of animals will carry a genetic mutation associated with a moderate phenotype such as seizures

-Some of the learning tasks will involve food restriction that is not harmful but may result in weight loss

-Some learning tasks will involve inducing a memory by delivering a mild foot shock, which may cause transient moderate pain and discomfort

-For testing audiogenic seizures, animals will be exposed to a loud sound and may experience seizures that will cause transient moderate discomfort

In all testing, attempts will be made to minimize discomfort to the animals (i.e., using the minimum loudness of sound to reveal seizures), and they will be humanely killed at the end of the experiment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Severities for all procedures will be mild to moderate. The majority of animals will experience an actual severity of mild.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project requires molecular investigation of neuron function and investigation of complex behaviours. To date, experimental systems that allow this are limited and must involve the use of vertebrate animals.

Which non-animal alternatives did you consider for use in this project?

Mammalian cell lines and primary neuronal cultures were considered.

Why were they not suitable?

Neuron cultures provide a limited picture of the molecular function of the brain, and they cannot be used to model behavioral changes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals has been estimated based on the breeding and maintenance required to maintain multiple transgenic lines, and experimental group sizes needed for molecular, biochemical, electrophysiological and behavioral assessment of multiple rodent models of autism. The groups sizes have been determined based on statistical analysis of previously published research, estimates from previous and current studies performed in our group, and collaborators performing similar experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In order to reduce variability, and thus number of animals required, all experiments will be performed according to the ARRIVE guidelines. As such, genetically altered animals will always be tested alongside unaltered wild type animals that are matched for age and gender, and treated groups will always be compared to vehicle controls. Furthermore, experiments will be performed blind to genotype by using a code (e.g., A or B) to identify each animal. This code will be kept by a person who is not involved in the experiment. Groups will be assigned in a randomised fashion.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

For experiments involving brain tissue and brain slices, experimenters can and often do use material from one animal for multiple experiments. In many cases, two researchers prepare hippocampal slices from the same animal to perform their experiments. In

addition, multiple researchers use the same brain lysate samples to perform Western blot experiments for proteins of interest.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will breed animals with disruptions in genes that have been linked to neurodevelopmental disorders. We do not expect these animals to suffer any adverse effects from these genetic alterations. The animals will be killed by humane methods and their tissues used after their death to study biochemical and electrophysiological changes of interest. In some cases animals may be given drugs to study the effects on biochemical/electrophysiological changes but it is not expected that this will cause any adverse effects other than mild clinical signs (such as decreased appetite or lower levels of activity). In some cases, we will test animals for their performance on learning and memory tasks or test them for audiogenic seizures. This will be necessary to reveal potential benefits from our drug treatments. In all testing, attempts will be made to minimize discomfort to the animals (i.e., using the minimum loudness of sound to reveal seizures), and they will be humanely killed at the end of the experiment.

Why can’t you use animals that are less sentient?

A major goal of this research is to identify and correct neurological processes that are disrupted in neurodevelopmental disorders using pharmacological and genetic strategies. The use of the genetically altered mutant rodent models is critical to investigating this question, because they effectively model human mutations that affect brain function. In the majority of our studies, we will use genetically altered mice in order to examine an intact mammalian nervous system. However, we will also need to use genetically altered rat models because they will allow us to assess more complicated cognitive disruptions that are difficult or impossible to observe in mouse models. For example, in behavioural paradigms, rats are more flexible in response to novel situations and have extensive social interactions - two domains specifically affected in autism spectrum disorders.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animals in these studies will be cared for by trained staff within a well-resourced and well- equipped modern animal facility that contains individually ventilated cages (IVCs) and barrier systems to maintain specific pathogen-free (SPF) status/health. Environmental enrichment will be provided.

Mice will be carefully monitored and if there is any evidence of suffering greater than minor and transient or in any way compromises normal behaviour they will be culled using schedule 1 methods.

Animals tested for audiogenic seizures (AGS) will be analyzed using multiple methods in order to identify a signature that is predictive of seizure onset. Identification of this signature may then be used for further studies rather than the behavioral expression of seizures themselves.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will stay up to date on best practice guidelines set forth and regularly updated from the NC3Rs website (Guidance on the Operations of ASPA - https://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about the advances in the 3Rs by attending informational events provided locally and provided by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs).

147. Acute and chronic kidney disease: translational studies using the pig

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

(i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

(ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

kidney, AKI, therapy, porcine, physiology

Animal types	Life stages
Pigs	juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Using a pre-clinical, translational animal (porcine) model, this project will develop and test therapies to either prevent, treat or benefit recovery from acute kidney injury (AKI), thereby blunting transition from acute-to-chronic kidney disease

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acute kidney injury (AKI) is an important cause of in-hospital morbidity and mortality in humans, in both acute medical and surgical specialties. For example, approximately 20% of patients undergoing cardio- thoracic surgery develop AKI, as do between 20-30% of patients in intensive care units and between 5- 15% of all admissions to hospital. AKI is ‘silent’ with no obvious symptoms until severe and currently there are no treatments for AKI. Therefore, at present, treatment can only be supportive, with optimization of fluid balance and blood pressure, avoidance of nephrotoxins and institution of renal replacement therapy where necessary. There has been no significant improvement in the outcome of AKI over the past 3 decades, yet AKI costs the NHS approximately £1.2 billion per year in added costs (increased length of stay, supportive care). Pigs are very similar to humans, especially their kidneys.

Indeed pig kidneys are more similar to human kidneys than the kidneys of non-human primates such as chimpanzees or marmosets. Therefore commercial pigs can be very useful and translational models when assessing kidney health and disease. Here, we have established a porcine model of AKI to undertake work relevant to the three stated objectives in this licence.

What outputs do you think you will see at the end of this project?

Other researchers in the UK: the results and data will be published open-access in peer-reviewed clinical journals. Therefore, the information will be disseminated amongst scientists and clinicians primarily within the nephrology community but will also be of interest to cardiologists and intensivists, since the risk of AKI is high in these settings. Within all these areas there is considerable interest in an early biomarker for AKI as there is no specific treatment for AKI, only supportive therapy. AKI can have a rapid onset and lead to patient deterioration very quickly. For these reasons, AKI is of huge importance to the NHS in the UK but also for health care services worldwide. Potential early detection alone (for example of metal elements in urine) or when such results are input to an iphone and coupled to machine-learned algorithms for early eALERTS of AKI, as has been reported recently, then we may be able to say we have a genuinely ‘early warning system’ for AKI.

International researchers: Globally, 85% of AKI occurs in developed countries. One output from our projects could be an easy-to-use point-of-care-test (POCT) that could be used in resource-limited areas. This would enable early triage of patients to larger hospitals and/or early, easily-administered supportive care, such as fluids and avoidance of nephrotoxic drugs (surprisingly common). A cheap, easy POCT test for AKI therefore has the potential to be distributed globally.

Technology: To date, there are very few hand-held systems for measuring metals in watery environments, with high sensitivity. Most require larger machines. The new technology we aim to develop as part of this licence when used in a novel health-care setting will be of interest to many environmentalists, geochemists working in the field. In

theory, the technology could be applicable in many other settings and be easily transportable.

Who or what will benefit from these outputs, and how?

In this project, using a porcine model of AKI we hope to discover underpinning mechanisms of acute and/or chronic kidney disease that can be exploited first to; 1) guide development of new therapies that may be used to either prevent AKI. This would be important for those individuals with no known underlying renal disease but whom are undergoing procedures that have a known risk of causing AKI e.g. patients admitted for cardiac surgery or into intensive care units (ICU). The timeframe for these advances from underpinning knowledge to clinical translation would be 3-5years; 2) to develop a therapy or technique that could treat those individuals where AKI has already been diagnosed e.g. in the community or within hospital, but at present only supportive care is currently available e.g. by giving fluids. 3) benefit recovery of functional kidney tissue after AKI so as to mitigate the known risk of transition from acute-to-chronic kidney disease. Secondary objectives of the research will be to consider new biomarkers of acute or chronic kidney disease or of successful recovery of kidney function. All research will be conducted alongside clinical colleagues to ensure that the research is grounded in translational science and that any positive outcomes maybe communicated immediately to the relevant (renal consultants and healthcare workers) in the shortest timeframe.

How will you look to maximise the outputs of this work?

There are few groups in the UK doing this type of work. The PPL holder has lines of communication with these other groups and in the past has shared tissues/expertise, as to be expected with such work. Any animal work will be published in peer-reviewed journals and presented at relevant conferences such as The Renal Association, The The Amercian Society of Nephrology etc..

Species and numbers of animals expected to be used

- Pigs: 128

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Upon deciding on the most appropriate animal model in which to satisfy study objectives then we will first consider if in vitro techniques are available and suitable – we are currently developing a number of these (e.g. microfluidics of drug uptake in kidneys). The proposed work will be conducted alongside these approaches. I always consider if less sentient animal models (e.g. flies, worms, fish) are satisfactory for study hypotheses, although for

genuine pre-clinical work in relation to renal physiology these are rarely appropriate. Mice, rodents have only residual renal functional capacity and fail to respond to AKI in a manner similar to human. The pig is the preclinical animal model of choice for testing therapies that may rapidly move to a randomised controlled trial. For example, in terms of general metabolism (but equally applicable to organ function) the pig represents one of the few species of animal that naturally carry a significant amount of cholesterol within the low density lipoprotein (LDL) fraction (similar to humans). For more acute studies in which the similarity between porcine and human physiology is the primary outcome then certain breeds of commercial pig, as supplied by an accredited supplier are suitable. Pigs can naturally develop renal disease, diabetes and heart disease, esp when fed high fat or salty diets. The majority of data generated is readily extrapolated to man, more so than many other animal models.

In order to examine specific mechanisms for the induction of disease then animal studies are advantageous, and whole animal physiology is important to characterise prior to experiment. Pigs can be fed westernised diets, are monogastric omnivores and actually develop all the symptoms and end- stage diseases that are important for this licence aims and objectives. Their size of organs in relation to body weight is near identical to human - this is an important consideration when studying physiology.

AKI is an important cause of in-hospital morbidity and mortality, in both acute medical and surgical specialties. Approximately 20% of patients undergoing cardio-thoracic surgery develop AKI, as do 5- 20% of admissions to general intensive care units. Thus in order to replicate this in an animal model we need to place the animal under general anaesthetic and reversibly occlude both renal arteries for a period of time that parallels clinical practice i.e. patients undergoing cardio-thoracic surgery. Direct occlusion whilst under anaesthetic ensures precise occlusion of the blood flow to the kidneys, as happens clinically with low blood pressure. Furthermore, longitudinal sampling, as enabled by using large animals, ensures less biological variation (within animal variance only) and thus greater statistical power to reduce animal numbers used in experiments.

Typically, what will be done to an animal used in your project?

Typically for any individual animal.

Objective 1-3: The pig will be acclimitised for one week. During this time the animal will remain in a pen, eating standard diet. **A preventive treatment may be given prior to surgery either via diet, water or by injection (Objective 1).** On the day of surgery, the pig will be randomised (sealed envelope) to study group and sedated for intubation and general surgery. Male or Female (usually female) pigs (50-65 kg; Canberra 12 [large white/duroc/landrace]) will be anesthetized for general surgery using an intramuscular dose of buprenorphine (0.05 mg/kg), ketamine (5 mg/kg), and detomidine (0.1 mg/kg) followed by intravenous alfaxalone (0.7–2.4 mg/kg), dosed to effect to aid intubation. Anaesthesia is maintained with isoflurane (1–2% in O_2) and ventilated using a tidal volume of 10–12 ml/kg. A venous catheter is inserted for supportive fluid delivery and vascular

access. Baseline blood samples are taken at this point. A bladder catheter is inserted for baseline urine collection. A midline laparotomy allows visualization of both the right and left kidneys, whereupon one or both common renal arteries are cross-clamped for variable time (commonly 40 mins; but previously from 20 - 60mins) using atraumatic vascular clamps. At time zero, the clamps are removed. At this point, some pigs may remain on the surgical table and physiology followed for a period of upto 4-6hrs, followed by euthanasia (i.e. non-recoverable surgery, **Objective 2**). These experiments will examine physiological events before, during and immediately after AKI. Objectives 1 and 2 will be conducted according to procedures outlined in Protocol 1.

In other pigs which usually have had bi-lateral renal artery occlusion - as with uni-lateral occlusion the remaining healthy kidney in situ accomodates for the reduced function of the previously occluded kidney, the incision site is closed, the bladder catheter (12 Fr) remains in place for upto 72h and pigs are recovered to a pen, thereafter for upto 8 weeks of age (**Objective 3).** Postoperative analgesia is maintained with buprenorphine every 8 h for the first 24 h and thereafter every 12–24 h, including tramadol (4 mg/kg im) as required. This objective will determine how pigs recover from AKI and can be used to test interventions where AKI has occurred in the community for example i.e. no preventive measure could be given. It is often inappropriate to only occlude one kidney as this rarely happens in the real world. Pigs recover fine from such transient occlusion so long as the period of occlusion does not extend beyond 120mins. Objective 3 will be conducted according to procedures outlined in Protocol 2.

All pigs will at a minimum have a baseline paired blood and urine sample, and thereafter be sampled at intervals to 8 weeks of age following prior protocols. At 48-72h, the bladder catheter is often removed to prevent infection and blockage. Thereafter spot samples of urine can be obtained by free catch. In some pigs at a sufficient post-operative recovery time (e.g. at least 5-7days), a percutaneous right kidney biopsy may be obtained with ultrasound guidance and pigs lightly sedated. At study end, all pigs will be humanely euthanised and tissues and biofluids collected as per standard protocols.

Standard measures of AKI are routinely measured in blood samples such as serum creatinine (µmol/l) and urea (mmol/l) will be measured by autoanalyzer (RX-Imola, Randox, Co. Antrim, Northern Ireland, UK) with typical interassay variation of <5%. Osmolality (Osmomat 030, Gonotech) and blood gas analysis will also often be conducted (pH, pO2, pCO2, HCO3, glucose, lactate, blood urea nitrogen, haematocrit (%) & haemoglobin (g/dL), Na+, K+, ionized Ca2+, Cl-; Siemans EPOC blood analysis system) as per individual study protocols. A typical blood sampling regime might be (10ml at each timepoint) 0h (i.e. baseline) and subsequently at 2, 4, 6, 8 24, 48h, 3d, 5, 7, 14 days and every 7 days from three to eight weeks of age.

Standard measures of AKI are routinely measured in urine such as albumin (g/L), creatinine (µmol/l) and their ratio will be determined. Urine samples (2ml at each timepoint) will be withdrawn at 0h (i.e. baseline) and subsequently at 2, 4, 6, 8 24, 48h, 3d, 5, 7, 14 days and every 7 days from three to eight weeks of age. Urine biomarkers of renal injury

will often be measured using commercial, porcine- specific ELISA assays. Nephrocheck© may also be conducted on urine.

What are the expected impacts and/or adverse effects for the animals during your project?

To minimise suffering: All experimental animals will be monitored for good general health, according to an adverse effects monitoring sheet developed by our unit. Each animal with the exception of some sham-controls on occasion, will be placed under anaesthetic for all surgical procedures. This involves sedation, anaesthetic, handling and/or restraint. Any invasive procedure that is likely to cause pain or temporary discomfort, will always be accompanied by appropriate use of analgesia. Animals will be monitored for weight loss over sufficient periods of time although this is not to be expected. Abnormal behaviour is to be expected temporarily post-surgery, and particularly after induction of AKI (moribund, lack of engagement). Such effects last for up to 24h. Analgesia will be used at appropriate intervals to manage any estimated pain, as previously established in association with a european diplomat in anaesthesia.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mild to moderate for all procedures. All animals will experience at least a degree of sedation (mild) or general surgery (non-recoverable or recoverable). All animals recovering from surgery are expected to experience moderate severity of procedures, managed using appropriate anaesthesia and monitoring.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project aims to investigate a specific disease affecting a single organ whose dysfunction affects all other organs of the body. Studying physiology is best achieved using live animals, as in vivo physiology better reflects the variable physiological responses observed in humans experiencing similar conditions. Cell-based models or less sentient animals like flies, worms or fish do not adequately reflect the human response to kidney injury. The pig is considered the most appropriate pre-clinical animal model to meet study objectives. For example, pigs are ideal for testing preclinical therapies with respect

to kidney function whereas rodents allow effects of aging or genetic manipulation to be explored. The data obtained will inform computational models of kidney function so that fewer animals are used for similar work in the future.

Which non-animal alternatives did you consider for use in this project?

Non-invasive technologies are available for studying isolated kidney cells and include cell lines from multiple species. In other less sentient animals, then whole kidney responses to lack of blood flow are not able to be recorded and they are not replicable in the clinic.

However, where possible we will develop other in-vitro methods such as cell culture. Indeed, with colleagues we have developed a primary culture-based model of kidney development (HK2 cells) and it is envisaged that we will also try to adapt this method for investigating isolated renal cell responses to certain conditions encountered during IR-injury such as hypoxia. Where appropriate we will also use in silico modelling of kidney function.

Why were they not suitable?

Non-invasive technologies are available for studying isolated kidney cells and cellular response to isolated hypoxia or malnutrition for example. These technologies are considered and used by the PPL holder as appropriate. However, for final pre-clinical discovery science or testing with a view to translation to clinical practice then studying in vivo in the live animal is required. The primary effects to be examined in this licence relate to a whole body physiological response and for this reason it is important to utilise the most appropriate animal model and then establish the causative factors in the circulation that may not be present in a culture dish, such as hormones or anti-sense RNA. With isolation of such putative causative agents then future in-vitro studies are much more realistic. This is not done lightly and all other options are always considered. One strength of the current programme of work is that we will combine these technologies with techniques that are not able to be performed, ethically, on humans e.g. kidney biopsies and for whom it is ethically difficult to acquire control samples e.g. kidney biopsies from individuals with normal kidneys, as a standard biopsy has a 1:3000 risk of fatality. Equally, the size of pigs allows for repeat blood sampling and kidney biopsies to be taken with relative ease and low impact on the animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of pigs allocated per group is ordinarily based on previous studies involving (n = 5-8 pigs/group) and consideration of the variation in primary outcome. Sham-controls are always run alongside treatment groups but numbers are kept to an absolute minimum due to low between-animal variability in kidney function. Where appropriate factorial designs are used to maximise the power of the experiment, plus longitudinal designs to minimise within-animal variation. Groups are determined by randomization (e.g. sealed envelopes) to minimise any subjective study bias. Typical number per group are n = 3/4 for controls and n = 6/8 for treatment groups. These numbers take into account either binomial outcomes (disease noted, yes/no) or quantitative outcomes such as the increment in serum creatinine with AKI.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Aspects of study design and outcome are always taken into account and are pre-determined in discussion with animal bioscientists. Power calculations are performed as necessary, based on aforementioned known variabilities in outcome. The PPL holder helps many other scientists with their statistics and uses the EDA for teaching undergrads study design. The PPL holder holds 'Stats club' sessions on an as needed basis for staff and undergrads. As an example, factorial designs are used to achieve greater statistical power with lower numbers of animals per group. For quantitative data, I always conduct a power calculation to determine optimal sample size and keep animal numbers to a minimum whilst maintaining statistical significance (alpha set at 0.05) and study power (>80%). For example, using a statistical program for performing power calculations then: assuming a 4 group factorial study using one sex, the effects of injury (injury, yes/no) and pre-treatment with a drug (yes/no) are tested for their interaction, then to observe the least significant practical effect size of 20% (e.g. a 20% change in renal creatinine clearance) with a residual mean square (variance) of 15% then we would require n=6 animals per group (total animals=24). Over the course of 3 years, using a similar design it is possible that we will repeat such a design, testing different drugs or different outcomes on at least 4-5 occasions, hence total study number for this PPL could number 128 over three years.

Using the above example, but conducting a split-plot design in which each animal served as its own control (i.e. unilateral renal occlusion) and assuming less (50% less) variance within kidneys than between, then we may be able to reduce animal numbers to n=4 per group or a total number of 16 animals. These figures depend on the experimental design and the variance associated with the outcome of interest. We will however, always make our best effort to accommodate these factors in order to keep animal numbers to a minimum. For example, we have previously justified the use of experimental controls (but not full sham-operated animals) with a small study comparing sham vs. un- anaesthetised controls on our main experimental outcome. Often, however, sham-surgery is required to replicate the minute variations in animal experience, environment, physiology as elicited by the experimental procedures such as surgery.

Experimental variance will be kept to a minimum by: Randomisation of animals to treatments, Explicit inclusion and exclusion criteria, avoidance of anaesthetic agents that may interfere with study outcomes. For each and every experiment, as part of good laboratory practice, we write an experimental protocol which includes: a statement of the objectives, a description of the experiment to be conducted including treatment groups, animal numbers and experimental material, an outline of the analysis and principal experimental outcomes.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will only purchase the required number of animals as per the study design. For any testing studies then we will always consider a it a pilot project, not using more than 3 animals for testing. For any new protocol then the design will include step-testing of one animal, then a further animal, then if all outcomes are met a full study design. Any tissue required by others will be biobanked and shared as the PPL holder has done in the past with multiple groupd. Evidence for this can be provided on request.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Upon deciding on the most appropriate animal model in which to satisfy study objectives then we will first consider if in vitro techniques are available and suitable – we are currently developing a number of these (e.g. microfluidics of drug uptake in kidneys). The proposed work will be conducted alongside these approaches. I always consider if less sentient animal models (e.g. flies, worms, fish) are satisfactory for study hypotheses, although for genuine pre-clinical work in relation to renal physiology these are rarely appropriate. Mice, rodents have only residual renal functional capacity and fail to respond to AKI in a manner similar to human. The pig is the preclinical animal model of choice for testing therapies that may rapidly move to a randomised controlled trial.. For example, in terms of general metabolism (but equally applicable to organ function) the pig represents one of the few species of animal that naturally carry a significant amount of cholesterol within the low density lipoprotein (LDL) fraction (similar to humans). For more acute studies in which the similarity between porcine and human physiology is the primary outcome then certain breeds of commercial pig from accredited suppliers will be used. Pigs can naturally develop renal disease, diabetes and heart disease, esp when fed high fat or salty diets.

The majority of data generated is readily extrapolated to man, more so than many other animal models. All pigs will be sourced from an accredited, commercial source with high health status and from a strain likely to be free of malignant hypothermia.

Why can’t you use animals that are less sentient?

In order to examine specific mechanisms for the induction of disease then animal studies are advantageous, and whole animal physiology is important to characterise prior to experiment. Pigs can be fed westernised diets, are monogastric omnivores and actually develop all the symptoms and end- stage diseases that are important for this licence aims and objectives. Their size of organs in relation to body weight is near identical to human - this is an important consideration when studying physiology.

Upon deciding on the most appropriate animal model in which to satisfy study objectives then we will first consider if in vitro techniques are available and suitable – we are currently developing a number of these (e.g. microfluidics of drug uptake in kidneys). The proposed work will be conducted alongside these approaches. I always consider if less sentient animal models (e.g. flies, worms, fish) are satisfactory for study hypotheses, although for genuine pre-clinical work in relation to renal physiology these are rarely appropriate. Mice, rodents have only residual renal functional capacity and fail to respond to AKI in a manner similar to human. The pig is the preclinical animal model of choice for testing therapies that may rapidly move to a randomised controlled trial. For example, in terms of general metabolism (but equally applicable to organ function) the pig represents one of the few species of animal that naturally carry a significant amount of cholesterol within the low density lipoprotein (LDL) fraction (similar to humans). For more acute studies in which the similarity between porcine and human physiology is the primary outcome then certain breeds of commercial pig as provided by accredited suppliers are suitable. Pigs can naturally develop renal disease, diabetes and heart disease, esp when fed high fat or salty diets. The majority of data generated is readily extrapolated to man, more so than many other animal models.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The PPL holder will always consider new, better methods that help reduce any animal suffering and improve study outcomes. For example, we altered our anaesthetic regime in consultation with the NACWO, named vet and veterinary consultants when it was apparent that a different regime produced better study outcomes (e.g. improved post-operative recovery). Equally, we sought to reduce unnecessary harm to sham-controls (i.e. no mid-line laparotomy) when it was apparent they were similar to other controls that had undergone full sham-surgery. Anaesthetic monitoring by a competent person when the pigs are under general anaesthesia will ensure welfare is not compromised. For example, this will be done by pedal reflex, corneal response and depth of breathing (mechanical ventilation at all times) to ensure appropriate depth of anaesthesia. A ventilator will help control the depth of breathing this will reduce any incidence/ likelihood of inappropriate

depth of anaesthesia and welfare issues. Post-operative monitoring follows a standardised process as developed in consultation with the NACWO, named vet and anaesthetic consultants. Pain management involves a pain-scoring table. If possible, animals will be trained to pee on demand. Animals will either be housed in groups to help reduce stress or if that is not possible and they have to be housed individually, then they will always be in sight and sound of other pigs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow any published guidance for large animals that may improve our study outcomes such as published by LASA or NC3Rs or the Home Office. For example, in regard to aseptic surgery then the guidelines as outlined in "Guidance principles for preparing for and undertaking aseptic surgery" (LASA pdf) will of course be followed.

We will ensure only the minimum number of animals are used as per trial design (pilot study or full experimental trial). The applicant routinely conducts power calculations on sample size (Genstat, VSNi Ltd) for all studies using a priori data.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The PPL holder is a member of an AWERB committee and regularly hears about updates on NC3Rs, from colleagues and the NC3Rs website. Any improvements will be implemented without hesitation. For example, we altered our anaesthetic regime in consultation with the NACWO, named vet and veterinary consultants when it was apparent that a different regime produced better study outcomes. Equally, we sought to reduce unnecessary harm to sham-controls when it was apparent they were similar to other controls that had undergone full sham-surgery.

148. Reproductive toxicology

Project duration

5 years 0 months

Project purpose

- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Toxicity, Reproduction, Toxicokinetics, Safety

Animal types	Life stages
Mice	juvenile, adult, pregnant, neonate
Rats	juvenile, adult, pregnant, neonate
Rabbits	adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to assess the safety of substances that may be administered to Man. The studies performed will be designed to reveal any effects on mammalian reproduction.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is generally accepted that the way in which a material is metabolised within a living body has a significant effect on how it works and its potential toxicity. Consequently, for the majority of substances forming part of the work performed under the authority of this

licence, it is imperative they are tested on living animals in order to assess for toxicity of tissue, organs and systems (e.g. the cardiovascular, respiratory and reproductive systems) following single or repeated administration. Unfortunately, the use of alternative methods, including the use of dead animals, at this moment in time cannot generate relevant data to support the submission of safety data to international regulators. Alternative methods such as in-vitro techniques will be used as much as practicable to supplement the work involving protected animals.

The testing of Pharmaceuticals as part of the safety evaluation process for clinical trial and marketing applications will generate data which will be used to make decisions on whether a candidate can be progressed to human studies. Similar assessments are also required for food and drink additives and supplements and novel foods to ensure their safe use in the human population. Study reports will be included in regulatory submissions to allow regulatory authorities (e.g. EMA or FDA) to make judgments on whether to permit clinical studies or to licence a drug. Guidance issued by the Home Office in 2005 recognises that the justification for animal-based regulatory toxicology and safety testing is the need for regulatory authorities to have sufficient information to assess the risks to which humans are exposed when test substances are manufactured/produced, transported or used. Thus, the cost-benefit assessment performed under the 1986 Act assumes, in the case of regulatory toxicology and safety testing that the principal benefit is the facilitation of sound regulatory decisions rather than the utility or profitability of the end-product. Study data will also be used to alert regulatory authorities to any potential safety concerns for substances already being administered/consumed by Man. Data are also used to inform starting or limit doses and to identify parameters which should be evaluated in clinical studies. Without these studies progression of new medicines to early human studies and to patients/marketing could not occur.

Data generated from studies performed in support of discovery and early candidate selection will be used to allow decision making on whether to develop new drug candidates and, with other preliminary studies will help to define doses for subsequent studies. For compounds which progress, these studies will be included in regulatory submissions as supporting studies. Early indications of toxicity liabilities will allow early detection of unsuitable candidates and thus development of the candidates which are more likely to progress, thus reducing associated development costs and animal use. Studies performed on ‘tool’ or competitor compounds may help to improve candidate selection and reduce resources and animals used in e.g. progressing candidates from a pharmacological class or chemical series that has inherent toxicology liabilities associated with it. These studies will help to prevent sub- optimal compounds from progressing through drug development.

Some ‘enabling studies’ will typically be performed to provide data in support of dose selection,typically tolerability and/or toxicokinetics, and allow other parts of a safety evaluation programme to proceed in an appropriate timescale with best possible outcomes.

The benefits of studies which address issues arising during the production, manufacture and marketing of substances, for example changes in production processes/locations, or impurity specifications will be very specific to the issue under resolution for example: allowing a more efficient/cost effective development process to be used, allowing use of a batch of material with a characterised impurity profile.

What outputs do you think you will see at the end of this project?

The development of safe substances such as pharmaceuticals and food stuffs (additives, supplements, ingredients and novel foods) and immune therapies (for example), is essential in protecting human health. The non-clinical studies permitted by this project are, therefore, crucial to generate data and reports for submission to regulatory authorities to guide safe product development and inform scientists of possible adverse effects.

The expected output from this project will be robust data, in the form of study reports, to enable the safety assessment. Study reports will be included in regulatory submissions to allow regulatory authorities (e.g. EMA, FDA, HSE or EFSA) to make judgements on whether to permit clinical studies to proceed, to licence a drug or to grant a marketing application.

Who or what will benefit from these outputs, and how?

Safety assessments detailed in this licence will ensure the safe use and management of products for human consumption/administration, particularly in pregnant women. In the short-term, this is achieved by providing high quality data to guide decisions regarding the suitability and the safety of the product for further development. Ultimately, these outputs will ensure that humans are not exposed to hazardous products in foods or drinks, and in the case of pharmaceuticals and therapies, the data generated will support the development of safe new medicines designed to improve the health and quality of life of patients.

How will you look to maximise the outputs of this work?

While much of the substance specific data generated is covered by confidentiality agreements, work on novel biomarkers, refinements in methodologies, protocols and techniques that permit a reduction in the number of animals required for specific study designs or to achieve specific end points are freely shared and discussed at Scientific conferences and other forums (e.g. attendance at regular NC3R meetings).

Although most studies will require the use of concurrent Controls to provide contemporaneous data for direct comparisons (to represent animals undergoing the same regulated procedures, administered the same vehicle etc), data generated from Control animals is held in reference databases to provide information relating to normal biological variation, thereby enhancing interpretation of study findings.

Species and numbers of animals expected to be used

- Mice: 30,000
- Rats: 30.000
- Rabbits: 2500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is generally accepted that the way in which a material is metabolised and distributed within a living body has a significant effect on how it works and its potential toxicity. Unfortunately, at this time, effects on complex interacting biological systems cannot yet be replicated using in-vitro or ex-vivo methods (non-animal models). Consequently, the use of animals is still an essential part of safety testing.

All studies performed under this licence will use the least sentient species practicable for achieving the study objectives. The majority of cases will involve the use of rats and mice. In embryofoetal development studies only, a second mammalian species is required and in terms of practicality and availability the rabbit is generally accepted as the preferred non-rodent species.

Studies conducted under this Licence will allow exposure to mature adults and all stages of development from conception to sexual maturity.

Typically, what will be done to an animal used in your project?

The studies performed under this licence will use either pregnant or non-pregnant animals. Non pregnant animals are typically used on preliminary studies designed to assess the tolerance of the test material or to establish suitable dose levels for use on main studies which generally assess effects on the foetuses following exposure via the placenta.

Animals will be acquired from designated suppliers. In many cases the supplier will provide animals which have been mated at specific timepoints so that the period of gestation is known. In pregnant rats and rabbits dosing will typically start on day 6 of the animal gestation and continue to at least day 17 (rats) and day 19 (rabbits) or in some case up to the day before necropsy. Such studies will be terminated on day 20 in the rat and day 28 in the rabbit.

The test material will typically be administered using the intended therapeutic route, which is typically oral gavage, but may include injection into a muscle, vein or subcutaneous (under the skin).

During the course of the study the animals will be well cared for and will be closely monitored for reactions to treatment. Blood samples may be taken periodically to assess

proof of exposure of the test material. Other end points will also be included as required to address specific concerns (e.g. functional observation tests).

At the end of the study the animals will typically be euthanised and a necropsy undertaken. This is essential because it is important to establish if the internal organs and or foetuses have been affected in any way and this can only be achieved by examinations by qualified personnel (Foetal Morphologist/Pathologist).

What are the expected impacts and/or adverse effects for the animals during your project?

It is anticipated that some animals will lose weight, or at least fail to gain weight at a rate consistent with normal weight gain. This may be attributed to a reduction in food consumption (also a potential adverse effect), but may be present even with normal food consumption.

Animals will be closely monitored for signs of discomfort and particularly signs of pain. Any animal showing such signs will be closely monitored and will be inspected by a Vet if considered necessary. No animal will be allowed to endure pain for long periods and remedial action which may include euthanasia will always be taken.

Where pregnant animals are used it is anticipated that foetuses exposed to the test material via the placenta may experience clinical reaction that may affect their well-being. Pregnant animals will be carefully monitored throughout gestation for signs of that could indicate that pregnancy is being affected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Our experience in this field of research has determined that reaction of no more than Moderate severity will be experienced. Indeed, our records show that overall, the majority of animals (mice, rats and rabbits) experience Mild severity only, circa 70% with 30% reaching Moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is generally accepted that the way in which a material is metabolised within a living body has a significant effect on how it works and its potential toxicity. Also the potential direct effects of a test substance on living tissue are often different to that predicted by ex-vivo or in-vitro methods. Whilst consideration will always given to the use of non-animal alternatives, currently there are no alternatives that can totally replace the use of animals. Non-animal alternatives i.e. those that use cells and computers for example are typically supportive/preliminary studies that provide supportive data for pre- clinical regulatory studies and for this reason, studies involving the use of animals remain a regulatory requirement.

Which non-animal alternatives did you consider for use in this project?

Sponsors will typically utilise non-animal alternative assays as part of the development programme for a substance (e.g. in silico modelling and in vitro metabolism studies). Such assessments would not be conducted under this project, however, where available, these data would be used guide the selection of the most appropriate species and to confirm the in vivo study design (e.g. to confirm the species which show similar patterns of metabolism to those expected in man).

Whilst information from these alternative assays will contribute to the overall safety assessment, they cannot replace the need for in vivo studies at this time.

Why were they not suitable?

The use of alternative methods, including the use of dead animals cannot, at this time generate relevant data which supports the submission of safety data to international regulators. The fundamental aspects of the safety data we require involves assessing physiological, behavioural and biochemical effects following the administration of a test substance and this can only be achieved by using live animals.

Alternative methods such as in-vitro techniques will be used as much as practicable to supplement the work involving protected animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The establishment maintains detailed records pertaining to the numbers of animals used on projects each year, as well as the number of study types undertaken. By analysing annual trends and having knowledge of industry requirements, it is possible to project the

number of study types we will undertake during the life of this licence thus enabling the estimation of the number of animals required.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The minimum number of animals will be used, recognising the fact that reduction is not achieved by using too few animals to achieve the objectives of the study. For Regulatory studies, guidelines require the number of groups and animals per group to be adequate to clearly demonstrate the presence or absence of an effect of the test substance; core study designs are based on international guidelines where these exist. Otherwise reference is made to internal guidance on study designs to provide the optimum number, balancing the need to achieve study objectives while avoiding excessive animal use. Project specific variations are used as required. The core study designs have been used extensively under the previous project licence and in other facilities and we have a track record of successful submissions and ability to eliminate unsuitable compounds. They are generally in line with those used throughout the pharmaceutical industry.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Statistical input is sought, where appropriate, to strengthen the overall scientific quality and relevance of the studies to be performed, with power-sample size calculations performed for specific studies if necessary to determine group size. For preliminary studies, small groups are acceptable because of the potential use of overt toxicological endpoints. Where group sizes are sufficient (rodent studies), data from definitive toxicity studies are analysed statistically.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In most cases the studies will be performed using internationally recognised guidelines (e.g. ICH and OECD) which define the most appropriate methods and scientific models to use.

The majority of animals used throughout this project will be rats and mice. On occasions however, and when scientifically justified, the rabbit will be used. The selection of an appropriate species will be a combination of ethical, scientific and practical consideration.

The species chosen will be the lowest neurophysiological sensitivity to achieve the objectives of the study and one which responds to the primary pharmacodynamic effects of the test substance; In most cases, this will be the rat or mouse.

Most pharmaceuticals need to be tested on a second species, with the second species being a non- rodent (CPMP/ICH286/95) modification). The selection of a suitable non-rodent species is of paramount importance as it will maximise human safety, clinical benefit and animal welfare.

The methods (procedures) used will be validated, well established and commonly used within the research community. The administration of test substance, removal of blood, collection of urine for example will cause no more than transient discomfort or distress. Any signs of distress will be carefully monitored including the onset and severity of treatment related effects. Appropriate and swift action will be taken to avoid any undue pain or distress.

Why can’t you use animals that are less sentient?

The studies performed under the authority of this licence are primarily to assess the toxicity of materials when exposed to foetuses as well as the parenteral animals. The use of immature life stages or species that are less sentient than these simply would not allow us to achieve the objectives of our studies.

Similarly, the duration of the studies which, in the case of multi-generation studies may be several months and studies often requiring behavioural assessments simply does not allow them to be performed under general anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures required will be undertaken by competent staff, each having undergone extensive training. Where necessary, current, industry accepted techniques for dosing and blood sampling for example, will continue be refined under separate authority i.e. under another project licence, thus ensuring existing methods remain the most appropriate for minimising pain, suffering and distress to the animals.

Animals will be monitored immediately after undergoing a procedure for any signs of adverse effects and will continue to be monitored at appropriate intervals until it is deemed that further observations are not required.

Animals will be habituated to procedures whenever considered necessary i.e. when it is deemed that by habituating animals to a procedure distress will be reduced. Similarly,

where appropriate animals may be trained to perform certain tasks thereby, minimising distress by removing the need to restrain an animal or involve direct contact during its performance.

In addition to this, the company is an industry leader in the application of microsampling techniques for obtaining blood samples in toxicology studies, which not only reduce the volumes required but also reduce the severity of the sampling procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The volumes administered to the animals and the volumes of blood taken will be in compliance with industry accepted guidelines. The primary guideline used is:

"A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes"; Karl-Heinz Diehl, Journal of Applied Toxicology J.Appl. Toxicol. 21 15-23 (2001).

The company is an industry leader in the application of microsampling techniques for obtaining blood samples in toxicology studies, which not only reduce the volumes required but also reduce the severity of the sampling procedures.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Staff maintain a proactive attitude towards the 3R's. Several members of staff are already participants in Industry Forums which discuss the 3R's in some detail and report any advancements to relevant persons. These advancements will be discussed further and implemented into our standard practices, where appropriate. The company is an established leader in the development and application of the 3Rs in toxicology studies.

149. The reciprocal interaction of inflammation and cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Inflammation, Bladder, Colorectal, Cystic Fibrosis

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand how inflammation predisposes to cancer development and how cancer causes tissue damage and inflammation, which can in turn promote progression of cancer in a feedforward loop. We will focus initially on two common cancers: colorectal cancer and bladder cancer, generating new animal models for these conditions where necessary.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Inflammation and cancer interact to cause much suffering and death in the UK.

Bladder Cancer (BC) is the third most common cancer in men; is increasing in incidence;

is one of the most expensive cancers to treat; and affected individuals have some of the worst cancer experiences. The median survival for metastatic BC is 6-9 months and survival rates are not improving.

Inflammation is the immune response to tissue damage or infection. However, many cancers drive inflammatory responses (“the wound that does not heal”) and inflammation is a known driver of cancer.

In addition to well-established causes of bladder cancer such as smoking and occupational exposure to aromatic amines, chronic inflammation has been postulated as linked to the development of bladder cancer. Epidemiological studies have shown a link between risk of BC and a history of urinary tract infections (UTIs). Other triggers of chronic bladder inflammation include schistosomiasis, human papillomavirus infection and chronic chemical and mechanical irritation to the urinary bladder. The chronic inflammatory microenvironment in bladder cancer is believed to be linked to initiation, progression and treatment response. The chronic inflammatory microenvironment includes tumour associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), mast cells, neutrophils, lymphocytes as well as inflammatory cytokines such as TNF alpha. However the molecular mechanisms underlying the link between the chronic inflammatory microenvironment and tumour initiation and progression remain to be determined. Elucidating these mechanisms can potentially lead to treatments targeting the inflammatory pathways believed to be driving initiation and progression of bladder cancer. The zebrafish model is ideal due to the capacity for *in vivo* live imaging of pre- neoplastic cells during tumour initiation and the interaction with inflammatory cells.

Colorectal Cancer (CRC) is the fourth most common cancer in the UK with survival rates for advanced cancers of only 10% at 5 years.

CRC is particularly common in people with cystic fibrosis (CF) - a well documented inflammatory condition. This is particularly true following solid organ transplantation. CF adults have an unexplained
~5-fold increased risk of GI tract cancers, rising to 30-fold in transplanted CF individuals.

Tissue inflammation and genomic instability are recognised as key enabling characteristics underpinning all cancers, facilitating subsequent acquisition of multiple cancer "hallmarks”. Mutagenic DNA damage is caused by pro-inflammatory production of reactive oxygen species (ROS) and, reciprocally, DNA damage is known to promote chronic inflammation. The cystic fibrosis transmembrane conductance regulator (CFTR) is important for ion transport at all epithelial surfaces, including the bowel. While CF is principally characterised by recurrent pulmonary infection and inflammation leading to tissue damage and respiratory failure, intestinal disruption involving chronic inflammation is a frequent feature. In mice, conditional inactivation of CFTR in myeloid-derived cells slows resolution of inflammation, indicating that CFTR has a poorly-understood role in resolving inflammation. CFTR has been identified as a candidate driver gene for colorectal cancer (CRC) in several forward genetic screens in mice, with GI tract-specific knock-out of CFTR resulting in increased adenocarcinoma in models of CRC, suggesting CFTR acts as a tumour suppressor gene in intestinal cells.

Many unknowns remain relating to the reciprocal interaction of cancer and inflammation, with a pressing need for new models to deliver new understanding. Since many immunomodulatory therapies are already available, we hope to be able to repurpose these for prevention of cancer.

What outputs do you think you will see at the end of this project?

This work will provide a new understanding in the pathological mechanism of cancer initiation and progression, as well as new information on how cancer causes tissue damage and inflammation, and vice versa. In addition to identifying the cellular drivers of several cancers, we will generate information about the function of genes known to, or suspected to, cause cancer-associated inflammation. The expected outputs and benefits of our work are:

A greater understanding of the cancer-inflammation axis and the factors triggering it. We will identify cellular and genetic drivers of the disease which will have a medium- to long-term benefit as they will provide invaluable new targets to underpin the rational design of new therapeutic approaches and to validate in patients.

A list of potential therapeutic targets from a suitable vertebrate animal model of inflammatory carcinogenesis.

An opportunity to develop more nuanced zebrafish models of cancer. Using transparent fish with a variety of visually trackable immune cell markers that switch on/off in response to stimulus could provide better understanding of the fundamental question is inflammation driving cancer, or is it a consequence of cancer?

The data generated by this programme of work will be disseminated to the scientific community via publication in international peer-reviewed journals, as well as presentation at (inter)national conferences.

Who or what will benefit from these outputs, and how?

Short-term

Development of new models, approaches and knowledge will benefit the scientific community and may lead to advances in areas unanticipated at the start of this work.

Medium-long term

Patients with cancer will ultimately benefit, although it is our aim that we would prevent cancer in patients with inflammatory syndromes such as cystic fibrosis. It is hoped that a greater understanding of the molecular mechanisms driving bladder cancer as well as understanding the link between inflammation and bladder cancer will lead to novel targeted treatments to improve outcomes for patients.

How will you look to maximise the outputs of this work?

We will liaise with patient organisations through bodies such as CRUK, Fight Bladder Cancer Charitythe Cystic Fibrosis Trust to ensure that our outputs deliver meaningful impact for patients. We aim to publish in open access journals and in preprint services such as bioRxiv.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 22000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Zebrafish are a vertebrate species with a comparable immune system to that of mammals. All major components of the innate immune system are present and share homology with their mammalian counterparts. Zebrafish are an excellent model for these studies. The larval stages are virtually transparent, with zebrafish lines, such as Casper, readily available for visualisation of early internal tumour development. The transparent nature of the larvae makes them uniquely suitable for the visualisation of the cell biology of inflammatory and oncological responses within a living host.

Zebrafish are readily amenable to genetic modification, with gene knockdown and overexpression strategies easily achieved.

Typically, what will be done to an animal used in your project?

A proportion of fish experiments are performed on larvae before the onset of independent feeding – these larvae are not considered protected by the Animals (Scientific Procedures) Act.

During embryonic and larval stages, the zebrafish will be anaesthetised and fixed in agarose to enable imaging using fluorescence microscopy at approximately day 3, day 7, day 14, day 28 and day 56.

In order to determine the outcome of manipulation of the inflammatory or oncogenic environment on cancer development we will need to generate adult zebrafish bearing oncogenic or pro-inflammatory mutations or transgenes. This will lead to small numbers of animals developing cancer during their lifetime. These animals will not be subjected to further procedures, but will be observed for the development of tumours, then killed by a schedule 1 method and examined histologically.

What are the expected impacts and/or adverse effects for the animals during your project?

As above, some animals will develop tumours. Our focus on the urinary and GI tract will mean that most tumours will be in these organs. One of our zebrafish lines will have homozygous inactivation of the p53 gene which can cause peripheral nerve sheath tumours, angiosarcomas , germ cell tumours and leukaemia beginning to develop at 4 months of age and become externally visible at around 7 months of age. We will inspect zebrafish daily to identify any adverse effects which will result in schedule 1 killing.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

In total, approximately 37% of zebrafish with homozygous deletion of tp53 develop externally visible tumours by 12 months of age. We anticipate that we will classify these animals at moderate severity. All other animals will be mild or subthreshold.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Inflammation and cancer are complex processes involving many cell types, mediators and different tissues. When both of these processes occur in one disease, an in vivo model is required to look at the interaction of these two complex processes - tumourigenesis and inflammation. Using the zebrafish we are able to efficiently perform genetic manipulation that is not possible in any other system in which the innate immune system can be adequately studied. Where data are available in the public domain (eg. Bioinformatic and RNA seq data) or where studies have been done in other systems, these will not be repeated in our model.

Which non-animal alternatives did you consider for use in this project?

Cell culture

Bioinformatic analysis (in silico) Lab-on-a-chip

Why were they not suitable?

None of these is suitable, due to the requirements to model complex interactions between tissue types.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of adult zebrafish required are calculated based on a requirement to have enough healthy adult fish at any one time to generate the larvae required for experimentation. Although large numbers of larvae can be obtained on one spawning, fish cannot then spawn for 2 weeks, meaning a rotating stock of fish is needed to ensure embryo supply. To minimise animal use, once experiments have been performed sperm will be frozen (for future IVF) and stocks will not be maintained.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use the NC3R's Experimental Design Assistant to consult on sample sizes for individual experiments to ensure only the minimum number of animals required is used. We have performed pilot experiments in order to ascertain key timepoints. Exploratory experiments have been carried out where possible on larvae prior to independent feeding, to refine the hypothesis-driven experiments. We have searched on-line databases to identify bioinformatic data which will allow us to narrow the range of hypotheses and sometimes to refute them without experimental work. For an important problem such as cancer, these animal experiments will allow us to deliver important advances.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have sufficient pilot data to perform a priori power calculations to calculate group sizes. We will not initially exceed the predicted group sizes required to detect a 25% difference with 80% power (alpha of
0.05 as per convention). We will increase group sizes only if greater power is required, if the variance is greater than in pilot studies, or where the biological effect to be detected is less than 25%. Where we obtain data over time from the same animals, we will use statistics appropriate to repeated measures. The use of the same animals over time greatly reduces the animal number required (compared with groups of animals sacrificed at each timepoint as occurs in many mammalian studies). Where possible adult fish stocks will be shared with existing PPLs, further reducing the number of stock fish required.

Zebrafish are highly fecund, so we are able to obtain large numbers of embryos <5.2dpf from a small number of parents. We will use pilot studies to optimise the number of animals used in this project. We will share tissue where it is feasible to do so.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Fin clipping and gamete expression methods rarely involve discomfort, but for all manipulative procedures animals are suitably anaesthetised (primarily for restraint). Anaesthetic is delivered by immersion (so no instrumentation is required) using an anaesthetic protocol suitable for the procedure and following best practice.

The methods for generating mutants and transgenics are optimised to minimise numbers and adverse effects are minimal.Generation of mutant/transgenic animals with tumour propensity will be based on well established techniques for mutation and transgenesis, and the animals will not suffer directly due to the techniques. However, the appearance of tumours might lead to suffering and needs to be closely followed.

Where feasible, early genotyping using a zebrafish embryonic genotyper (ZEG) system will reduce the number of larvae raised as we will only raise those of the required genotypes.

Why can't you use animals that are less sentient?

Zebrafish are the organism with the lowest neurophysiological sensitivity that is suitable to study the innate immune system and address our proposed aims. They have a number of advantages for these studies, including optical transparency, genetic tractability and extensive genomic resources.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are in communication with other cancer research groups worldwide to ensure that we follow current best practice for identifying tumours in fish to ensure that they do not suffer.

For any experiments performed beyond the onset of independent feeding, larvae will be monitored regularly, with only the healthiest larvae being selected for imaging studies, and individual experiments will be evaluated before further larvae are used in this protocol. The advantages and risks of this approach will be evaluated after each larva is used.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To the best of my knowledge there are no published best practice guidelines for the zebrafish work in this project license. However we use philosophies of experimental design advocated by the likes of Festing and Wurbel in order to refine our experiments.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am on the Impact Working Group of the NC3Rs and am in regular contact with the team. I also have access to a NC3Rs regional programme manager with whom I will consult as well as attend relevant workshops/seminars to learn about the latest developments in the 3Rs. This keeps me updated with evolving 3Rs best practice.

150. Nucleic acid sensing and interferon-regulated genes

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

immune system, immunisation, virus infection, cancer, nucleic acid sensing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to gain new knowledge on how the immune system is activated and on how it controls virus infections and cancer as well as the response to vaccines.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Viruses cause diseases ranging from the common cold to AIDS, and pose the risk of pandemic outbreaks. Our body is protected from infections, including viruses, via a

protective network called the immune system. The immune system can eliminate viruses, and it is therefore important to understand how the immune response is kick-started upon infection.

The first step is that the cells in our body recognise the presence of a virus. We know that cells have specialised proteins called receptors that detect viruses. However, how these antennas sense viruses is largely unknown. By investigating the mechanisms of detection we hope to understand how the immune response is initiated during virus infection.

One of the hallmarks of the anti-viral immune response is the production of a group of molecules called interferons. The name stems from the property of interferons to interfere with and block the replication of viruses. Interferons achieve this by instructing cells to switch on their antiviral defences. Interestingly, interferons are not only essential as central players in antiviral immune responses. They are also produced during vaccination and are necessary for the development of protective immunity. Moreover, interferons are involved in cancer and may help our immune system to fight malignant cells. It is therefore important to study the effects of interferons. These new areas of research hold great promise for the development of new vaccines and novel cancer treatments. We want to obtain a better understanding of the underlying biology, which will be required for the development of new medicines.

What outputs do you think you will see at the end of this project?

The primary output of this work will be new knowledge in the area of immunology. This new information will be made available via publications in peer-reviewed scientific journals.

Who or what will benefit from these outputs, and how?

The information we will generate will be of interest to immunologists, virologists and to biomedical researchers interested in vaccination, autoimmunity and cancer, both in academia and industry (benefits expected to be evident within 2-5 years). We have chosen our specific aims with a clear view of generating data that hold translational value and may at some later stage form the basis of clinical trials. We have interactions with pharmaceutical companies working in these areas to facilitate the translation of our work.

How will you look to maximise the outputs of this work?

Over the course of this licence, we will publish our findings in academic journals. We will only publish in open access journals such that anyone interested will have full access to our data. We will also present our work to the scientific community at conferences. Where appropriate, additional channels to disseminate knowledge will be used, including Twitter and public engagement activities. We make new genetically altered animal models freely available to the scientific community upon their publication. For example, frozen sperm from previously generated mutant lines has been distributed to >20 laboratories world-wide. Our group has many collaborations, for example with virologists and oncologists, to maximise benefits in these related disciplines. Unsuccessful approaches are typically the

lack of a phenotype in a genetically altered animal line and will be included in publications. Publications will follow the ARRIVE guidelines to optimise the reporting of information.

Species and numbers of animals expected to be used

- Mice: 12000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice will be used to research the biology of how immune responses are initiated. Adult mice are used because they have a fully formed immune system that is similar to that of humans. Mice are also used because of the availability of genetic tools.

Typically, what will be done to an animal used in your project?

We will breed genetically modified mice that lack immune genes or have mutated immune genes. The majority of animals will be humanely killed without undergoing procedures and tissue will be used for experimentation.

In addition, genetically altered or wild-type mice may be infected with viruses, receive vaccines and/or be implanted with cancer cells. These steps typically involve a single injection. At appropriate stages of the ensuing immune responses, mice may receive injections of drugs, such as those used in people, to block molecular pathways of interest. By subsequently comparing treated and control mice, we will learn how these molecular pathways impact immune responses. The measurements made may include asking whether mice are better or worse protected from a viral infection (e.g. Influenza A virus), or we may harvest lymph nodes from killed mice and examine the quality of the immune response upon immunisation.

What are the expected impacts and/or adverse effects for the animals during your project?

We will use genetically modified mice that lack immune genes or have mutated immune genes and expect that the genetic modifications will not cause any adverse effects.

The expected impacts on mice undergoing procedures will be similar to those experienced by humans having a viral infection or receiving similar treatments. In the infection and vaccination models, some mice may suffer adverse effects that include fever, weight loss and behavioural changes, which will typically not last longer than 24 hours. Immunisations may also cause some local irritation/inflammation, which typically self-resolves within 24 hours. Mice receiving cancer cells will develop tumours, with palpable tumours typically present for less than two weeks.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Subthreshold - 83%
Mild - 9%
Moderate - 8%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to breed genetically altered mice to obtain (from killed animals) primary cells and tissues for subsequent analysis.

We also need to use animal models of infection, vaccination and cancer because the immune response is a complex process involving many different types of molecular mediators, cells and tissues. This cannot be recapitulated outside an animal.

Which non-animal alternatives did you consider for use in this project?

We considered:

1. the use of immortalised cell lines;
2. the use of primary human cells and tissues; and
3. in silico modelling.

Why were they not suitable?

1. Immortalised cell lines are not always suitable because some immune signalling pathways are altered during the process of immortalisation. In addition, cell lines representative of some immune cell types do not exist or cannot be generated.

2. Primary human cells from healthy donors can be obtained routinely only from blood or skin. Whilst we use such human samples in some of our research, we cannot entirely rely on this, because some immune cell types and tissues (such as spleen and lymph nodes, and cells resident within these organs) cannot be obtained. For some of our research questions, human blood and skin samples cannot be used because of genetic heterogeneity and the lack of appropriate gene editing tools for human primary immune cells. Other tissues, such as tonsils, may be obtained from donors with certain disease

indications, and other organs may be obtained from cadaveric donors. We rejected these options because it is important for our research to experimentally perturb a healthy immune system and because of ethical, logistical and technical limitations.

3. We collaborate with in silico modellers; however, while such approaches are useful for prediction, they cannot test real biological mechanisms or functions.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers of mice have been estimated based upon the numbers of experiments required to test our hypotheses. These estimates have taken into account our experience from the last ~15 years and projected changes in project scope during the next five years. Animal group sizes are based on appropriate statistical estimates and prior experience of levels of variability.

Numbers of animals required for breeding protocols take into account animals generated with the wrong genotype as well as animals that are required for colony maintenance. Animal numbers listed in experimental protocols include those transferred from breeding protocols as well as those purchased from the core facility and commercial vendors.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

1. We have used statistical models to determine the minimum number of required animals.

2. We have designed experiments in such a way that many data points can be collected from the same animal. For example, we often obtain multiple tissues from the same animal.

3. Where possible, surplus cells and tissues will be frozen for future use. For example, we routinely aliquot and freeze bone marrow samples for multiple future experiments.

4. Where possible, we will use male and female animals, which reduces the number of surplus animals from breeding.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. We will use a breeding strategy - managed by staff trained specifically in maintenance and breeding of mouse colonies - that keeps the number of mice to a minimum.

2. Unwanted genetic changes will be prevented by regular crosses to a reference mouse strain.

3. We will bank sperm from our mouse strains. This will allow us to archive strains that, whilst valuable, are not currently needed for experimental purposes, rather than continue to breed them.

4. We use pilot studies with small numbers of mice, for example where we test new vaccines.

5. Fresh and frozen tissue will be shared with collaborators where appropriate, including both donations and receipts of material.

6. Procedures on animals will only be carried out if a strong hypothesis can be obtained from in vitro data, in silico predictions, clinical findings and/or genetic associations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We chose mice because this is the lowest species suitable to achieve our aims. As soon as the scientific objective of any procedure has been attained, animals will be killed, and specific humane endpoints will be applied throughout. Therefore, we will not cause lasting harm. We will use models of virus infection, vaccination and cancer that are most refined and cause the least possible harm:

We will interrogate the early stages of infection. At these time points, the innate immune system becomes activated and we will study this process. However, at these time points, virus replication has not yet resulted in tissue damage that causes profound disease. Animals will be killed before they reach this later stage.

Freund's adjuvant will not be used. This is a component of vaccine formulations that has been used in the past and caused adverse effects such ulcerations. We will use other adjuvants that do not cause adverse effects.

We will use tumour models that are easy to monitor and do not form secondary tumours (metastases).

Why can't you use animals that are less sentient?

The immune system in mice is similar to the one in humans, allowing us to extrapolate findings. This would be impossible using lower protected animals (such as zebrafish) or non-protected animals (such as fruit fly), because their immune systems are highly divergent from the one in humans, in particular in the aspects we are studying.

We need to use adult animals because the immune system is not fully developed at earlier life stages in mice. Our protocols cannot be carried out under terminal anaesthesia because mice cannot be kept alive for long enough under terminal anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will consider introducing genetic alterations only in the tissue of interest using 'conditional' genetic approaches.

We will increase monitoring of animals in cases of concern or doubt, such as in pilot studies or unimmunised control groups.

If multiple routes of administration are scientifically suitable, we chose the one that causes the least amount of pain and distress. Examples for this refinement may include intra-nasal administration instead of injection and addition of substances to the drinking water instead of repeated oral gavages. Footpad injections will not be used. Instead, we will use a refined model (hock injection) that is much less painful because animals do not have to walk on a swollen footpad, but achieves a similar scientific aim.

For tumour studies, we will explore in vivo imaging techniques.

We use anaesthetics and analgesics appropriate for mice by consulting the literature and the NVSs.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the 3R resources (https://www.nc3rs.org.uk/3rs-resources). We will further consult the PREPARE guidelines (https://norecopa.no/prepare) and the "Guidelines for the welfare and use of animals in cancer research" (British Journal of Cancer (2010) 102, 1555 – 1577). Our work will be done in accordance with the LASA aseptic guide (http://www.lasa.co.uk/pdf/lasa_guiding_principles_aseptic_surgery_2010.2.pdf).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We continuously seek to identify new methods for replacement, refinement and reduction. All lab members will regularly attend the animal facility users meetings to learn about the latest developments in the 3Rs. We will continue discussing our experimental approaches with facility NACWOs and NVSs, as well as collaborators, so that protocols can be constantly improved. We will regularly visit the 3R website (https://www.nc3rs.org.uk/the-3rs) to obtain the latest updates. We are closely following the developments in our area of research via publications and at conferences with a view to identify, and where appropriate implement, refinements.

151. Breeding, production, archiving and the application of assisted reproductive techniques of genetically altered mice

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- o Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Mouse, Genetically altered, Cryopreservation, IVF

Animal types	Life stages
Mice	adult, embryo, pregnant, juvenile, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This license will permit the creation, archiving and introduction of genetically altered animal models into a world leading academic research institute for the duration of 5 years and will facilitate the efficient and ethical management of each live resource throughout the duration of various research programmes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Key objectives can be summarised as;

1. The generation of up to 40 novel genetically altered mouse lines per year over the course of the license using appropriate methods via in-house genome editing services.

2. To maintain breeding colonies of genetically altered lines for distribution to various research projects.

3. To provide the application of assisted reproductive techniques for the purposes of efficient colony management and the introduction of new mouse lines into the Biological Services Units. Rederivation techniques will also be applied to protect and improve the health status of model organisms across the academic institute.

4. The archiving and if necessary, the distribution of roughly 100 mouse lines per year over the duration of the project.

5. Where appropriate services will also be available to the wider research community. Equal harm benefit analysis will be conducted for both internal and external service provision. Any external work will be included in the aforementioned capacity.

What outputs do you think you will see at the end of this project?

The outputs from this project will be the creation of new genetically altered mouse lines that will support a wide variety of research areas. We will also ensure that animals can be maintained. with a better health status because the procedures in this licence will enable us to significantly reduce pathogens in the colonies we hold within our establishment. In addition to creation and healthy maintenance of animals we can use this licence to cryopreserve embryos and sperm to ensure that genetically altered animals can be stored for future use without having to keep breeding animals.

Who or what will benefit from these outputs, and how?

The wider research community will benefit from the outputs of this project licence by having new genetically altered mouse colonies that enable them to carry out novel and cutting edge research.

Researchers will also use animals that are free from disease which is good for both welfare and scientific outcomes. Researchers will also benefit from not maintaining excess numbers of mice in situations where they can be cryopreserved, which is also good for reducing the overall numbers of animals used.

How will you look to maximise the outputs of this work?

We have a dedicated facility for the work carried out under this project licence that actively seeks both internal and external opportunities to maximise the outputs of this project licence. Where possible, work under this project licence will be presented at national or international conferences and would be likely to reflect refinements in practice that have been developed during the course of the licence. For example, under our previous licence, we disseminated knowledge on how to reduce the number of animals that are breeding in

facilities through effective use of cryopreservation. We will use good breeding practices deveoped under this licence as a way of guiding researchers working under other project licences in how to effectively maintain their animal colonies. To further maximise the outputs of the work, we would publish any findings that would promote good practice within the industry and would actively support the dissemination of information on unsuccessful approaches.

Species and numbers of animals expected to be used

- Mice: 45,500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project is providing a service to other Home Office licences projects that use genetically altered mice. We use mice because the are the most commonly genetically altered species that are used for biomedical research. We need to use mice from the point of fertilsation through to adulthood. For example, we carry out in vitro fertilisation (IVF) with sperm and eggs in order to create the genetically altered animals that we require and we maintain animals through to adulthood for use in scientific procedures in other project licences or for breeding purposes under this project.

Typically, what will be done to an animal used in your project?

The majority of mice (approximately 60%) used in this project will be bred and maintained and will have subthreshold or mild symptoms resulting from their genetic alteration. They will typically have small ear samples taken for identification and/or genotyping and maintained until they are transferred to another project licence or humanely killed typically before they are 12 months of age but a maximum of 15 months of age when essential for maintaining a genetic iine.

Some animals (approximately 25%) will be used for superovulation. Female mice (dams) will be given agents that cause them to superovulate (cause them to release more eggs during a single oestrous cycle. These mice will then either be killed for egg collection or mated to produce embryos.

Some animals (approximately 15%) will be used as embryo recipients. These will be female mice that will firstly be mated with a sterile male to make them pseudopregnant (phantom pregnancy). These mice will then have embryos surgically (or rarely non-surgically) implanted in them that have been created or modified in another mouse or by intro fertilisation (IVF). Once the litters have reached weaning age, the dams will be humanely killed.

A very small number (<2%) of mice will undergo vasectomy. These male mice will undergo surgical vasectomy to render them infertile. They will be used repeatedly for making female mice pseudopregnant and will be humanly killed when they are too large or old for this use.

What are the expected impacts and/or adverse effects for the animals during your project?

Due to the nature of this project, the anticipated adverse effects within this license are likely to be very mild i.e. through breeding and maintenance of GA mice and superovulation protocols. The creation of novel genetically altered lines can cause adverse phenotypes, but adverse effects that are more than mild are not expected. Surgical protocols (vasectomy and embryo transfer) will cause a short term discomfort for animals that should not require pain relief after one day following surgery. All mice on this project will either move onto another project licence, or will be culled by a schedule 1 method.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately 85% of animals are expected to experience a mild severity. Approximately 15% of animals are expected to experience a moderate severity.

What will happen to animals at the end of this project?

Used in other projects Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice can only be bred and genetically altered through the use of live animals. Creating genetically altered animals for use as models permits the study of the functions and interaction of genes across broad subject areas, such as Cancer, Cardiovascular, Diabetes and Neuroscience studies. This project provides a service to support the production of novel and/or disease free mice that have genetic alterations that are essential for research purposes.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives are not possible because of the nature of this project and so could not be considered.

Why were they not suitable?

They were not suitable because there is no alternative to live mice for breeding purposes.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals that will be used based on our experience under our previous project licence and the projected service provision over the next 5 years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This project does not require experimental design because the purpose is not to use animals for experimental procedures. However, some animals may be humanely killed for tissue only and used for experimental purposes during the course of maintaining mouse colonies.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The centralised production, breeding and archiving of genetically altered mouse lines provides an opportunity to analyse working practices in order to use the fewest animals to achieve the intended aims.

By exploiting transgenic technology including the recent advances utilising programmable nucleases such as CRISPR/Cas9, it will be possible to generate novel mutants much more efficiently. Increases in targeting efficiency will result in fewer founder animals and reduced time frames.

Archiving mouse lines will inherently reduce the numbers of animals required for any given project and when completed pre-emptively, will allow for efficient line removal and if necessary expansion.

The archiving of mutant lines will predominantly be accomplished through the cryopreservation of sperm, which is significantly more cost effective with regard to animal usage than cryopreserving embryos. Breeding and maintenance of mutant lines for distribution to various research projects will permit centralised colony management,

reducing the breeding and managed excess required whilst ensuring the best husbandry and genetic integrity.

Distribution of novel transgenic lines to the scientific community will potentially allow for a global offset against production rates by reducing the necessity to recreate lines elsewhere.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All of the animals bred and maintained under this project licence will experience only mild (or subthreshold) symptoms of their genetic alteration. The diseases or bodily systems that they will be used to study will vary widely and these mice will move to other Home Office approved project licence for the study of these diseases or systems.

Some animals will undergo surgical procedures for vasectomy or embryo implantation which will be of moderate severity. These are not complex surgeries and the adverse effects are expected to be short- lived. Wherever possible, males that are infertile as a result of genetic alteration will be used as an alternative to surgically vasectomised males.

Why can’t you use animals that are less sentient?

As this is a service licence we would be unable to use less sentient species. However when creating novel genetically altered mice there is a robust project review before work commences.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Using an already established system for providing intesntive care post-operatively we will ensure animals recieve high levels of monitoring at all stages. Where pain management is required this will be used. If possible, genetically sterile males will be used instead of surgically vasectomised males. We will also look to increase use of non-surgical methods for embryo recipients.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Guidance will be obtained from several resources such as NC3R's, Jax and Mouse Genetics Informatics publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Local NC3R's programme manager. Updates from Home Office, NC3R's, IAT and LASA. Regular review of publications associated with this work. Where there are advances which will benefit 3R's these will be assesed and utilised wherever possible.

152. Mechanisms of neural circuit development in health and disease conditions

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain development, Neuronal wiring, Gene function, Neurological diseases, Neurodegeneration

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand the physiological and biological mechanisms that guide brain development and maintain neuronal connections. We also want to understand the role of genes in these processes and how gene mutations lead to neurological disease conditions.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neurological conditions such as neurodevelopmental disorders and neurodegenerative diseases place a huge financial burden in our society, where societal and emotional costs are incalculable. To be able to find treatments for these debilitating conditions, we first need to elucidate the basic mechanisms that underpin the normal formation of brain structures and neural connections. We will then be able to examine how human conditions arise from mutations inside genes essential for these developmental processes.

What outputs do you think you will see at the end of this project?

Work carried out during this Project will result in outputs through the appropriate channels of conference presentation and publication (10+ and 5+ respectively), wherever possible in high impact and/or open access journals, where they will be freely accessible to other researchers, clinicians and industry.

Who or what will benefit from these outputs, and how?

Other researchers, clinicians and industry will be able to utilise our findings in developing their own research and biomedical applications. In general, the short-term (1-5 yrs) benefits will be to our and other academic researchers, including those associated with the clinic. In the medium/long-term (5-15 yrs) we anticipate our findings and approaches may influence perception, diagnosis and perhaps even treatment of human disease.

How will you look to maximise the outputs of this work?

We will establish collaborations with other researchers by sharing knowledge, experience and reagents. We will also work closely with clinicians and industry optimizing the chance of translational impact.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): 19,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are using zebrafish as sole vertebrate animal model because the embryos are spawn in the water (allowing collection without affecting adult mothers), the animas develop externally and are optically clear (allowing for observation of brain development with no intervention needed). We therefore make most of our observations without any need for surgery or other intervention giving discomfort to the protected animal. Most of our experimental manipulations will be done during embryonic and larval stages. This is a period when neuronal connections are rapidly assembled in the brain and the animal

shows behavioural responses. This allows us to investigate how these connections are established and what goes wrong when genes implicated in human disease are mutated.

Typically, what will be done to an animal used in your project?

An animal used in this Project will typically undergo or have previously undergone genetic modification. This is important for generating fluorescent reporter transgenic lines essential for monitoring the cell behaviour and function. We also plan to establish lines with mutations in genes previously found to be associated with human disease. This is an important part of our research in understanding the molecular and cellular mechanisms that are responsible for the disease.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used in this Project are expected to experience minimal pain and suffering as a result of our manipulations. However, in certain cases when a genetically altered animal is created to mimic a human disease condition, it is inevitable that fish might experience some clinical symptoms. We will aim to reduce such clinical symptoms by maintaining our genetically altered lines in healthy heterozygous forms. Occasionally, our experimental manipulations can also produce side effects. We will make sure that any individual showing signs of discomfort is monitored regularly and, if the symptoms persist, we will kill animals by Schedule 1 methods.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority (80%) of animals used in this Project will experience pain or distress that is no greater than mild. We anticipate that a maximum of 20% of animals used in this Project will experience a moderate severity. In the rare event that fish need to be kept until the end of a procedure, it is first monitored for sign of pain and/or distress (lack of swimming activity, appetite or sexual behaviour). Any individual presenting any signs of pain or distress that is not rapidly curable will be killed under Schedule 1.

What will happen to animals at the end of this project?

Killed
Used in other projects
Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

As the main goal of our research is the understanding of the cellular and molecular mechanisms required for development and function of the brain in the vertebrate embryo, alternatives not using animals at all are not yet available. The originality and strength of our study is the ability to follow neuronal cell behaviour and function in vivo, using imaging of fluorescent transgenic zebrafish. In vivo studies are the only way to identify the cellular behaviour at the source of developmental defects.

Moreover, using in vivo studies allows the examination of the behavioural manifestations of genetic lesions, allowing us to perform small molecule drug screening to identify compounds that could ameliorate the symptoms, offering opportunities for novel drug development.

Which non-animal alternatives did you consider for use in this project?

Some of the molecular candidates involved in the formation of neuronal circuits will be assessed in primary neuron culture. We are currently starting to develop these culture technologies, hoping to get these to be used for some of our scientific questions, instead of the fish.

Why were they not suitable?

We did search for more alternatives, using FRAME and other sources suggested on the NC3Rs website, without success.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimation of fish usage is non-trivial, since multiple confounding factors make simple calculations impossible, and instead force reliance on previous experience within the wider fish researcher community. The key point is that the overwhelming majority of our animal usage is in generating or maintaining genetically altered (GA) lines. In the first case, stochastic effects are important in affecting i) how many founder fish actually have germ-line integration of the transgene, and then transmit to the next generation, as well as ii) the extent to which positional effects alter the behaviour of the transgene from that intended. In both cases, a further stochastic effect that is of major significance is that of the unpredictability of sex ratios. Furthermore, for almost all work, the key issue is a regular supply of good quality embryos for experimental study; this necessitates maintaining larger

than minimal numbers of fish so that individuals are not usually used more than once per week. Over-usage would be problematic from a welfare point of view since it could well result in stress. We note that the harmless nature of the GA in the majority of the lines when kept as hemizygotes or heterozygotes goes a long way to reducing to a minimum the suffering caused. The numbers requested in this license for each protocol have been estimated in accordance with these principles.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Almost all (approximately 90%) manipulations and observations will be performed on zebrafish embryos or early larvae at stages prior to the stage when they are capable of independent feeding, and hence of stages that are outside of the Animal (Scientific Procedures) Act. However, in the remaining cases (approximately 10%) it will be necessary to rear experimentally manipulated fish after they become protected by the Act. Where possible our emphasis will be on assessing effects as early as possible, so that older, larval and adult stages will be only used where completely necessary.

We will take advantage of the optical clarity of fish larvae to allow the use of new microscopic methods to image brain development or brain disorder models. The aim of much of our work is to use transgenes that do not disrupt normal processes to report on those processes as they happen. So, although the animals are transgenic and therefore fall under the Act, they will not suffer in any way compared to non- GA fish. Creating stable transgenic lines is, by itself, a way to reduce the number of animals used. The establishment of healthy viable adults carrying a transgene allows us to reduce the number of embryos studied compared to the quantity we would have to use in a transient system.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of our animal usage will result from generation and maintenance of mutant and transgenic stocks. We will use the latest transgenesis/mutagenesis techniques so as to ensure that the minimum numbers of founder fish are generated, and will take advantage of developments in inducible gene expression (e.g. CreERT2) to ensure that transgenes are expressed wherever possible only when needed. Where possible, we will perform genotyping at the embryonic and larval stages to only raise fish of the required genotype, and where feasible we will use transient CRISPR mutagenesis approaches to minimise the need for generating and maintaining mutant lines.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use zebrafish as a model system during this Project. Fish are the least sentient of the vertebrate models suitable for genetic research. The Project will use the latest in vivo technologies used by other researchers in the field to minimise any animal suffering, such as well-established gene editing and imaging techniques. Our methods will allow us to generate transgenic lines reporting the expression of transgenes in specific cell types within the brain, and mutant animals carrying mutations in genes required for neuronal development. We also plan to use imaging techniques to monitor neuronal cell behaviour and function and assays to investigate the behavioural manifestations of these genetic manipulations.

Why can't you use animals that are less sentient?

We chose the zebrafish as sole vertebrate animal model because the embryos are spawn in the water (allowing collection without affecting adult mothers) and are optically clear (allowing for observation of brain development with no intervention needed). We therefore make most of our observations without any need for surgery or other intervention giving discomfort to the protected animal. We anticipate that the majority of our procedures will not exceed mild severity limit and will not generate pain or distress (a maximum of 20% of animals are expected to exceed that limit, and no animal will experience a severity above moderate limit). In the rare event that a fish needs to be kept until the end of a procedure, it is first monitored for signs of pain and/or distress (lack of swimming activity, appetite or sexual behaviour). Any individual presenting any signs of pain or distress that is not rapidly curable will be killed under Schedule 1.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In all our studies we will address effects as early as possible and we will use anaesthetics whenever appropriate. Non-invasive methods will be used wherever possible. As stated in Protocol 1, the majority of lines carrying mutations will be kept as healthy heterozygotes and are expected to only show phenotypes when homozygous. Therefore, this will reduce any potential suffering of animals. Any individual presenting signs of distress or pain persisting in the hours following each treatment will be culled under Schedule 1.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will constantly look for published literature to ensure experiments are conducted in the most refined way. We will use pilot studies to refine protocols being developed/modified in

the lab. We will refine the use of specific anaesthetics as their advantages and disadvantages are understood. Where revised methodology has become available, we will endeavour to update our protocols accordingly.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will have regular meetings with our regional program NC3Rs manager and attend meetings/events organised by the NC3Rs.

153. Development of an implantable device for the treatment of brain tumours

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Brain, Cancer, Technology, Therapy

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.
What's the aim of this project?

To develop and test an implantable device, which has been designed to deliver chemotherapeutic drugs for the treatment of brain tumours.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

With more than 11,000 new cases each year in the UK, brain tumours are a common disease which significantly impacts the life of affected patients. On average, only 12% of patients survive for five or more years after the diagnosis.
Depending on each individual case, brain tumours are treated by a combination of chemotherapy, radiotherapy and, if possible, surgical removal. A fundamental limitation for the success of chemotherapy is the blood-brain-barrier, a membrane that separates the sensitive brain tissue from the blood and any toxic substances that may be dissolved in it. It plays a pivotal role in protecting the brain from toxic substances but also significantly

reduces the amount of cancer drugs which can be delivered into a brain tumour through the blood. Additionally, reduced blood flow to solid brain tumours further complicates the process of delivering enough drug to have a positive effect. The highly toxic nature of chemotherapeutic drugs limits the volumes that can be safely given to a patient because they can cause damage in healthy tissues as well as the tumour being targeted.
A different approach for enhancing transport through the barrier is avoiding it entirely by delivering cancer drugs directly into tumours. Convection enhanced delivery, for instance, is a technique where a thin tube is inserted into a brain tumour through which a cancer drug solution is injected. By creating a constant overpressure of the solution, the drug is forced into the tumour which allows better drug distribution than simply soaking the tumour in drug solution. However, convection enhanced delivery is significantly limited by the tendency of the drug to flow back out of the tumour through the hole created to insert the tube. To avoid this problem we will develop a device that can be implanted inside a brain tumour and will deliver anti-cancer drugs without pushing liquid into the brain. The anti-cancer drug, dissolved in fluid, will flow into the implanted device through an input tube. Once inside the device, an electric current will be used to separate the drug from the fluid so that only the 'dry' drug is delivered to the tumour. This is known as iontophoretic drug delivery. The fluid, now cleared of anti-cancer drugs, can be taken away through an output tube. We anticipate that this approach will solve the problems caused by convection enhanced delivery.

Iotophoresis has been an active topic of research for over a hundred years and was traditionally used to increase absorption of a topically applied drug through the skin. This allowed for the safe and painless delivery of drugs, through tissues that are typically impermeable, without the need to puncture the skin with needles and potentially invite infection. Traditional iontophoretic devices consist of a counter electrode and a working electrode, the latter of which is separated from the target tissue by a reservoir of the chosen drug. When a current is applied across the electrodes the drug is driven out of the reservoir directly into the target tissue. Between 1900 and 2019 almost 2800 publications have been published on iontophoresis and in the year 2020 alone there were 20 papers. The vast majority describe iontophoresis as a method for moving substances through skin but we hope to show that is can be used to deliver drugs within the body, with great accuracy and in a way that doesn't increase local pressure.

What outputs do you think you will see at the end of this project?

The output will be an effective, safe implantable device which will deliver drugs directly into tumours. We will also publish our findings in scientific journals which will include details on our design process and experimental methods.

Who or what will benefit from these outputs, and how?

At the time of writing there is another device in development within our group which aims to utilise the same delivery method for anti-seizure medication into the brain to treat epilepsy. The lessons we learn from the development of the cancer treatment device, from the choice of materials to the implantation method, will feed directly back into the decision making process for manufacturing and testing the epilepsy treatment device and all future devices with similar function. We will also be able to compare the function and efficacy against devices that have been, and are being, developed by other groups around the world.

In the long term, the publications that arise from the data collected during this project are likely to show, for the first time, that drug delivery devices of this type can be used in the treatment of brain tumours.
Many anticancer drugs, including cisplatin, cannot penetrate the barrier that separates the brain from the blood and therefore cannot be utilised in this role. If we are able to show that our device can shrink tumour size or reduce toxic damage to non-tumour tissues above and beyond what we would see with the current best available treatment, then we can move forward with the development of a device for human applications. Direct delivery of anticancer agents into brain tumours has the potential to improve the poor survival outcomes associated with brain tumours that are difficult to surgically remove.

How will you look to maximise the outputs of this work?

We will use the data generated, both significant and non-significant, under this project license to inform a number of research papers that we aim to publish in high-impact scientific journals. This should ensure that the outcomes of this work are recognised by a multi-disciplinary audience.

This work is part of an Interdisciplinary Research Collaboration (IRC) involving researchers from five leading UK academic establishments. At every step of the process there is constant communication between the researchers and the clinicians ensuring that our results feed directly back into the hospital setting.

Species and numbers of animals expected to be used

- Mice: 600

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this study we will use adult mice with a well characterised background and no deliberate alterations to their genetic makeup. These mice are ideal for our studies because the tumour cells that will be grown under the skin come from mice with a similar genetic background. It is unlikely that the tumour tissue will be rejected by the host so there is no need to use mice with compromised immune systems in order to grow the tumours. The animal facilities at our establishment are very well adapted to caring for small rodents and no major special provisions will need to be made prior to our experiments.

We will use adult mice because the device we are developing will ultimately be used in humans. Testing its function in anything smaller than adult mice will likely prove irrelevant and make the surgical implantations unnecessarily difficult. Whilst it would be beneficial to test the device in a much larger animal (e.g. pig) we believe that the information we get from mice will be sufficiently translatable into the clinical setting.

Typically, what will be done to an animal used in your project?

Diagram showing the 3 scenarios likely to be experienced by the animals.

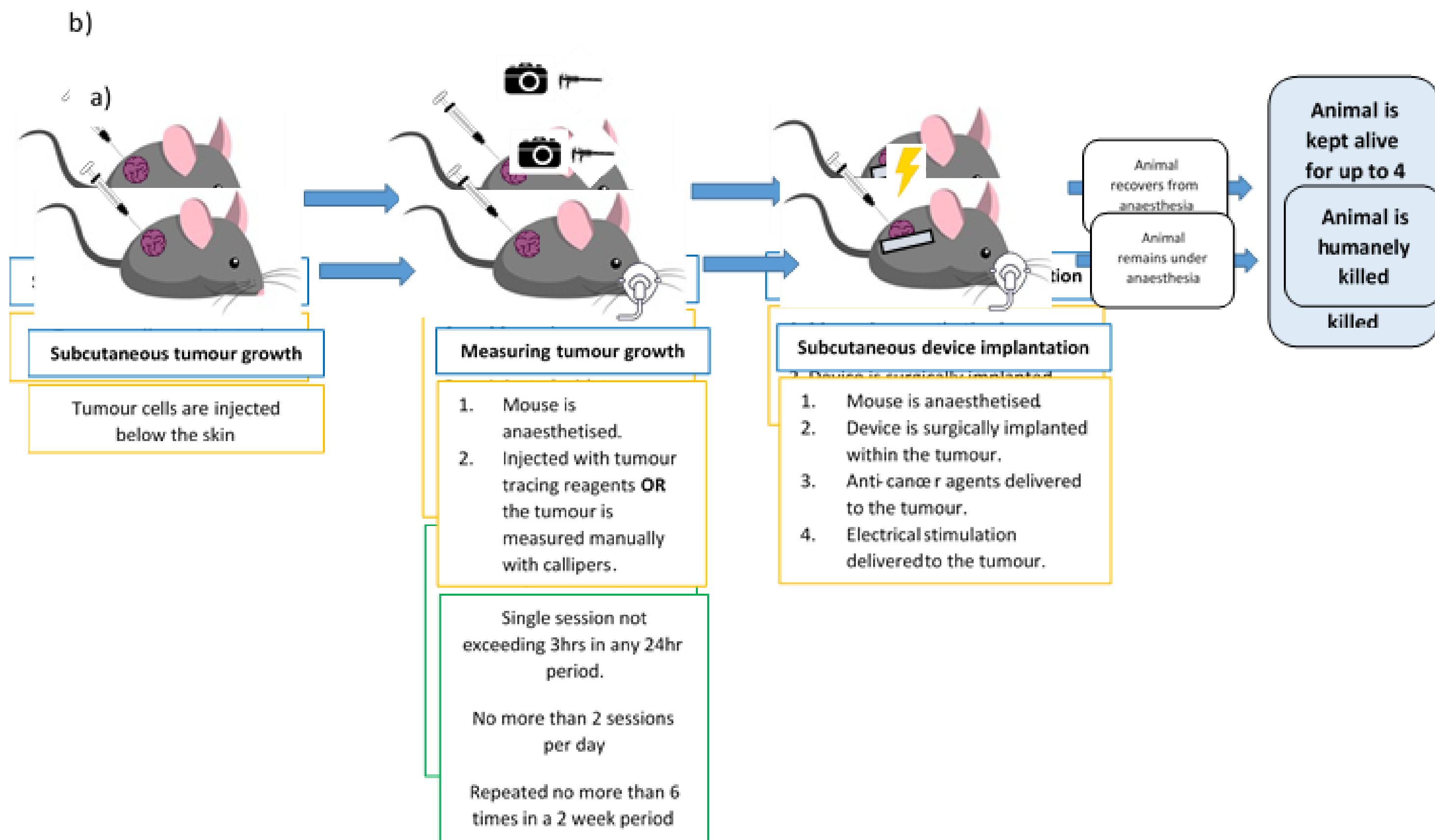

Figure a) Mice will have tumour cells injected subcutaneously which may require general anaesthetic and pain relief. The tumour will be allowed to grow to critical size and measured, using either specialised imaging hardware, for which the mice may be anaesthetised and injected with tumour tracking reagents, or manually with callipers, which may also require general anaesthetic for accurate recording. Once the tumour has reached the correct size the animal will be anaesthetised and the drug delivery device will be implanted within the tumour tissue. Anti-cancer agents and control substances may be applied to the tumour tissue alone or in combination with local electrical stimulation. Before the mice recover from the anaesthesia they will be humanely killed.

Figure b) Mice will have tumour cells injected subcutaneously which may require general anaesthetic and pain relief. The tumour will be allowed to grow to critical size and measured, using either specialised imaging hardware, for which the mice may be anaesthetised and injected with tumour tracking reagents, or manually with callipers, which may also require general anaesthetic for accurate recording. Once the tumour has reached the correct size the animal will be anaesthetised and the drug delivery device will be implanted within the tumour tissue. The animal will recover and spend up to 4 weeks carrying the inactive device under the skin before being humanely killed. We will analyse the samples from the implantation site to determine how the local tissue environment responds to the device over time.

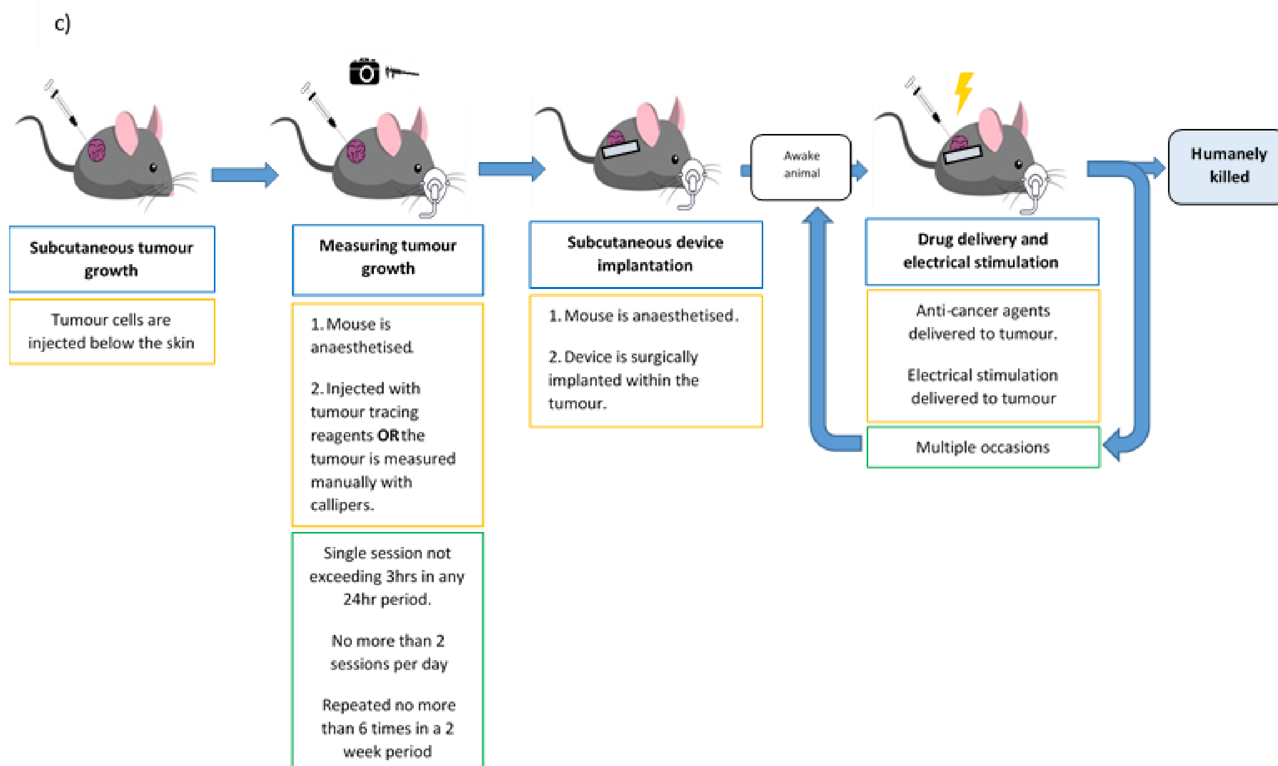

Figure c) Mice will have tumour cells injected subcutaneously which may require general anaesthetic and pain relief. The tumour will be allowed to grow to critical size and measured, using either specialised imaging hardware, for which the mice may be anaesthetised and injected with tumour tracking reagents, or manually with callipers, which may also require general anaesthetic for accurate recording. Once the tumour has reached the correct size the animal will be anaesthetised and the drug delivery device will be implanted within the tumour tissue, again requiring pain relief during recovery. After recovering from anaesthesia, anti-cancer agents and control substances may be applied to the tumour tissue alone or in combination with local electrical stimulation. The device will be operationally tested for a short time (up to one week) or over longer timelines (up to 4 weeks). At the end of the experiment the animal will be humanely killed.

Ideally, we would want to test the device in a brain tumour and brain with similar dimensions to that of a human, better replicating the target tissues and producing more relevant information. However, for the initial validation phases we believe that we can address gain valuable information using brain tumour cells grown under the skin of mice.

What are the expected impacts and/or adverse effects for the animals during your project?

We expect that the mice may experience mild and transient pain following surgery, however this will be countered using pain killers before and after surgery. Any further surgical complications, although unlikely, will be monitored for and advice will be sought from the Named Veterinary Surgeon should welfare concerns occur.
Humane endpoints refer to one or more physiological or behavioural signs that have been determined to signal when an experimental animal's distress should be terminated or reduced. This is typically achieved by ceasing a painful procedure, administering pain killers or humanely killing the animal.

We don't expect any serious adverse effects to the animal's general well-being during the tumour growth phase as tumour volume measurements will be carried out periodically to

ensure that humane endpoints are not exceeded. Most of negative clinical signs associated with tumours in mice present as weight loss and any mice that lose 15% of their pre-implantation weight will be humanely killed.

It is possible that adverse effects will occur in response to administration of the anti-cancer drugs, such as local tissue damage due to direct contact with the agents or an allergic reaction. Adverse effects that are expected include typical signs of toxicity associated with administration of anticancer agents. These may include acute effects such as inactivity or tensing of the abdominal wall to protect inflamed and painful organs. Adverse effects may also include chronic drug effects such as gut toxicity, caused when the toxic drug kills off the friendly bacteria that inhabit the gut and is most likely to manifest as weight loss. Chemotherapy is cleared from the body through the kidneys which can have a damaging effect on them, the resulting water imbalance that often presents as dehydration or, in some case, water retention. The extent of the damage is largely dependent on the individual dose, frequency and cumulative dose of the drug and also manifests as weight loss.

Platinum-based chemotherapeutic drugs such as cisplatin have been linked to permanent hearing loss in human cancer survivors and studies have shown that the same symptoms can effect mice enrolled on cisplatin treatment regimens. While not immediately threatening to the lives of the mice, it may interfere with their normal social behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Moderate severity, 100% of mice

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is critical that animals are used in the validation of this device because it is not yet possible to simulate the complex tumour environment outside of a living animal. Our aim is to get the device into the clinical setting where it can help patients with potentially life threatening diseases. Testing the safety of medical devices in animals before they are introduced in human clinical trials is required for both ethical and legal reasons.

Which non-animal alternatives did you consider for use in this project?

Several experiments have been conducted to gather as much preliminary information as possible without the use of live animals.

To measure the speed that the drug moves through the tissue after being released by the device, we cast the device in gel designed to be similar in density and mineral content to living tissue. The drug, bound to a coloured dye, was then released into the gel at varying rates and the speed that the drug travelled through the tissue was measured.
While the gel is a useful model for tissue, we also conducted similar tests using chicken breast and pig brain, purchased from the local butchers to get a better picture of how the drug moves through tissue in relevant biological material.

We have also run simulations, using computer software, that mimic the chemical and physical processes that will occur in the activated device.

Why were they not suitable?

Due to the delicate nature of the brain, the potentially damaging effects of chemotherapeutic drugs and the complex nature of cancer, there are no non-animal alternatives that would provide the peace of mind required to safely move the device into human trials. Non-animal alternatives are suitable for providing preliminary information which allows us to reduce the numbers of animals and refine the experiments but they do not satisfy the need to replace animals entirely.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Estimates of animal numbers are derived from a combination of 2 factors: our experience in developing implantable devices - particularly that derived from our previous licence, and our engineering knowledge on the typical number of new versions necessary to develop a successful device. Over the course of this licence, we expect to develop a progressively improved drug delivery device. From our previous experience, we expect to have to go through approximately 20 new versions of devices before arriving at the final design. From our experience, each new version requires approximately 25 animals to test and obtain decisive feedback on. While difficult to predict the exact numbers at this stage, this calculated estimation provides a good approximation to the number of animals we expect to use.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Where possible we intend to randomise the allocation of animals into either control or treatment groups to minimise systemic bias and improving the reliability of the results. We also intend to utilise blinding to ensure that the person analysing the results isn't subject to unconscious bias, further increasing reliability and reducing the requirement for repeated experiments. We will use an experimental design assistant (https://www.nc3rs.org.uk/experimental-design) and PREPARE guidelines (https://norecopa.no/prepare) to aid in planning the experiments. Our group constantly reviews the work in the field of bioelectronics to avoid unnecessary repetition of animal

experiments. We will use the online platform SyRF (http://syrf.org.uk/) to aid with our reviews.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pilot studies will be used to assess feasibility and outcome measures of the experimental plan, mainly regarding new treatments. Previous experience has shown that many potential problems can be identified and rectified in small pilot studies. This means that eventual definitive studies with larger groups of animals have a high probability of success, and large studies will not need to be repeated due to unforeseen study failures.
In order to ensure that the results are reported to the highest standard we will adopt the advice given in the ARRIVE guidelines. By doing so we hope to improve the reproducibility of our animal work.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

To test and validate the device in a relevant system we will need to generate a model of glioma. Ideally, we would want to test the device in a brain tumour and brain with similar dimensions to that of a human, better replicating the target tissues and producing more relevant information. However, for the initial validation phases we believe that we can address our objectives using brain tumours grown under the skin of adult mice.
In order to refine our protocols and reduce that chance of causing unnecessary pain to the animals we will work in a step wise fashion, collecting as much information from low risk methods, before progressing onto methods with a higher risk of complications. We will first test the device in mice that have been terminally anaesthetised, almost completely removing any pain, suffering or distress that could be experienced before the animal is humanely killed. Once we know that we can implant the device safely we will move into tumours that have been implanted under the skin for the next stage of testing. This will allow us to test the ability of the device to deliver drugs to a living tumour and its suitability as a long term wearable device without any risk of neurological complications.
The tumours will be implanted under the skin while the mice are under general anaesthetic with both pre-operative and post-operative pain relief. This ensures that animals don't experience distress but also improves the reproducibility as even small movements during implantation can have negative results on tumour uniformity.

Growing tumours in mice requires close monitoring as the cancers can expand rapidly and spread to other tissues in the body. To counter these risks, we will perform regular volume measurements using non-invasive calliper measurements or bioluminescent imaging . This allows us to take accurate measurements without using procedures anymore invasive than an injection and ensures that we don't allow to the tumours to grow beyond the humane

endpoints. Measurements will be taken under general anaesthetic to reduce movement and ensure the greatest accuracy.
Mice are a very useful species for cancer research because the disease is so well characterised after decades of research and publications. We know which procedures are likely to cause pain, which signs to look for and how to address any problems. By building on the work of others, we are able to learn from the lessons of the past and improve our methods for the benefit of the animals and our research.

Why can't you use animals that are less sentient?

Adult mice represent the smallest and least sentient animal that we could test our device, intended for human use, while still collecting relevant data. Using younger mice would likely make our surgical interventions more technically difficult than necessary and pose more risk to the animals.

We will use mice that have been placed under terminal anaesthesia for the initial stages of device validation, giving us the opportunity to test the function of the device in living tissues without the risk of complications that may cause suffering. As the device is being developed for chronic human use we will need to move testing into awake animals in order to show that it can be worn for extended periods of time, without malfunctioning or impeding the normal movement of the wearer.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The mice will be housed in groups, between three and four per cage, to stimulate their normal social behaviours. This is beneficial for the animal's mental welfare and, because distressed animals can become anti-social, allows us to visually assess their general health.

Following surgery the mice will have their surgery sites visually inspected and be weighed at least twice a day for the first 3 days, then once a day for as long as necessary. Weights and observations will be recorded on post-operative data sheets, ensuring a comprehensive account of each mouse's recovery. Mice that show signs of chronic distress such as erect hair, hunched posture, anti-social behaviour and weight loss of 15% of pre-treatment body weight will be humanely killed. We will also refer to the mouse grimace scale to recognise pain, assess its severity and take steps to relieve it.
Special nesting material and easy-to-eat food will be provided after surgery, as well as heat packs attached to the outside of the cage to aid recovery. Due to their large surface-area to body-volume ratio, small rodents are particularly vulnerable to hypothermia following surgery and the use of heat packs will prevent this.

We will utilise bioluminescent imaging to track the tumour growth over time. This technology requires the tumour cells to be genetically altered so that they carry the gene responsible for emitting light in fireflies. This gene will cause the tumour to glow inside the mouse, producing light that can be detected using a sensitive camera. Computer software will then determine the size of the tumour with greater accuracy than can be achieved using manual methods. The mice will need to be injected with a harmless reagent to stimulate the bioluminescence and briefly placed under anaesthetic for a clear image to develop, but the increased level of accuracy will make sure that the tumours aren't allowed to grow larger than necessary.

We will use non-tail handling methods, such as cupping and tube handling, (https://www.nc3rs.org.uk/mouse-handling-tutorial) and enrichment aimed at improving their quality of life. Every animal will be given at least a week to acclimatise to the new facility before any procedure is carried out. All our work is structured in a stepwise approach to only progress with interventions which are more likely to be successful. To achieve this aim, we will carry out initial work in mice under terminal anaesthesia.
To ensure that we are providing the best and most up-to-date care for the animals during the tumour implantation and growth stages, we will defer to the “NCRI Guidelines for the welfare and use of animals in cancer research” by P. Workman et al.
To deliver the best surgical outcomes for the animals and our research we will review the guiding principles listed in the "LASA Guiding Principles for Preparing for and Undertaking Aseptic Surgery 2017".

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will have regular discussions with the Named Persons and animal technicians to review current approaches and whether there are any new 3Rs opportunities.
We have subscribed to the NC3Rs e-newsletter, providing monthly updates that focus on funding opportunities, 3Rs events and publications.
We will also look at attending NC3Rs events and workshops to keep abreast of 3Rs advances and approaches.

Any new advances that may impact how we work or provide new approaches will be communicated with everyone working on this project.

154. Understanding epithelial tumour initiation

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Cancer initiation, Prevention, Epithelial cells, Development

Animal types	Life stages
Mice	adult, embryo, neonate, juvenile, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify the early cellular and molecular events that drive epithelial tumour initiation. This information will enable us to detect tumours early and prevent their progression.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is a devastating disease. Every day in the UK, 1000 men and women are diagnosed with cancer and 450 die from the disease. Progress has been made in developing better drugs to treat cancer. However, the most impactful intervention one can make for cancer patients is to detect the tumours early. The evidence is clear, the earlier a tumour is detected the higher are the chances of surviving the disease. For example, data from Cancer Research UK shows that breast cancer patients for which the tumours were detected early they have 90% chance of responding to treatment and surviving cancer-free for more than 10 years. In contrast, patients for which cancers were detected at a late stage of the disease they have less than 10% chance of surviving cancer-free for more than 10 years.

In aim of this project is to increase our understanding of how tumours develop. In particular we want to identify the early changes that occur in the tissues before tumours grow. This information will allow us to develop accurate early detection methods and hopefully new methods of how to prevent tumours from developing all together.

What outputs do you think you will see at the end of this project?

Advancing our basic understanding of how cancers develop. It is only now that we have the technical ability to study the early steps of tumour initiation. We will share any large datasets we generate in this project with the scientific community so it can be used by others in their research. Identification of new markers that can be used for the early detection of tumour initiation. These will be reported and shared through peer review publications.

Developing new genetically engineered mouse models that can be used to accurately study tumour development.

Patent and grant applications based on our findings to enable the development of early detection and therapies.

Who or what will benefit from these outputs, and how?

Scientists studying cancer biology. The large datasets and mouse models generated from this project will benefit their research. We deposit all our data in public repositories and also generate free user friendly websites that accompany the publications of these studies so everyone can access and benefit from the data.

Cancer Patients. The identification of new markers that can be used for the early detection of cancer will improve the clinical care provided for cancer patients. We aim to validate our findings in the mouse and pursue the translation of our findings as soon as possible.

How will you look to maximise the outputs of this work?

Data generated from this project will be presented at national and international conferences as well as shared through publications and pre-print outlets such as BioRxiv.

We will also continue to make user- friendly websites to access the single cell genomics data generated form the mouse studies.

It is important in science to also share unsuccessful approaches to minimize unnecessary animal use by others. We will aim to publish any unsuccessful and technically challenging approaches using BioRxiv so others can benefit from our experience.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Despite the significant improvement in cancer care over the last decades, we still have little understanding of the events surrounding tumour initiation. Most cancer studies to date, both in mouse and in human, have focused on characterising the tumour and how to shrink it. Now with the advances in sequencing technology we have the ability to study the very early steps of tumour development using genetically altered mouse models. This is important as it will reveal the earliest markers of cancer development, which can then be used for the development of: 1) early detection methods and 2) therapies that can prevent cancer development. These kind of studies are only feasible in the mouse, particularly when studying solid tumours (eg. Breast, Ovarian, Lung and Intestine). We will generate mice that are predisposed to developing cancer and investigate their tissues at the cellular level using novel sequencing technologies, prior to the detection of physical tumours. This analysis will reveal the very early cellular changes that occur in a tissue before tumours arise. It is impossible to perform these kind of pre-cancer studies in humans.

Typically, what will be done to an animal used in your project?

We will need to generate and maintain genetically altered animals.The majority of our work will be focus on tissue specific tumour initiation which will be achieved using tissue specific gene deletion/induction as this will minimise unwanted harm. The animals will be monitored for tumour development and in some cases treated with potential therapeutics. In addition, as most of our focus will be on the early steps of tumour initiation, we will have only a small proportion of our animals with fully developed tumours. In general mice in the project could be used for:

the generation of new or re-derivation of genetically altered animals obtained from public repositories or research labs.

the breeding and maintenance of genetically altered animals that can be used in the experimental protocols.

the treatment with tumour inducing substances or cell labeling substances or contrast agents for Xray/CT scaning using one the following methods (Steps 3-5) :

- oral gavage (a tube inserted via the mouth into the stomach to deliver a soultion).

13. subcutaneously (i.e under the skin).
14. intraperitoneally (i.e. into the body cavity).
15. intravenously (i.e into a vein)
16. implantation of a slow release pellet subcutaneously under anaesthesia.

It is not possible to fully predict the nature or severity of any potential defect from new genetic mutations. We expect if these new mutations predispose animals to tumour development, most of them (80-90%) will develop within 1year. In few animals (10- 20%), it may take up to 2 years of ageing for this new mutation to cause tumour development. Tumour inducing agents are highly mutagenic and hence increase the chances of tumour development. We expect 100% of mice treated with tumour inducing agents to develop tumours within 1 year. Transgene inducing/deleting reagents, in rare cases, may cause skin inflammation, thickening or flaking, hair loss or altered pigmentation, benign dermal cysts, skin erosions or tumours. General anaesthetic might be used if subcutaneous injections or implants are used (30% of animals). Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Surgical procedures will be carried out aseptically. In the unlikely event of post-operative complications, animals will be killed unless, in the opinion of the NVS, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

Mice might undergo direct injections of viral particles that can induce breast cancer.

More than 99% of animals are expected to make a rapid recovery. Analgesic agents will be used to minimise pain wherever possible. In rare cases (<1%) it is possible that adverse effects from anaesthesia could occur. The procedure is not expected to result in any frequent or chronic adverse effects. By removing the dead skin from the nipple non-surgically, we expect that the already low risk of infection will be further minimised. Mastitis is a possible adverse effect of injecting into the mammary duct. However, the risk of mastitis is greatly reduced by using a blunt needle which is as short as possible and is not inserted into the mammary gland. The fluid pressure is sufficient to carry the viral particles into the gland with the needle positioned at the tip. Mice that show signs of redness/inflammation (mastitis) following completion of an intraductal injection will be given a mild anti- inflammatory and monitored closely. Mice that display persistent mastitis (not resolved 72 hours after injection) will be killed by schedule 1 method. Viral constructs (for example constructs inducing oncogene expression) might lead to the development of tumours.

- Viral reagents might be delivered via inhalation to induce gene expression, deletion or editing directly in the lung.

Delivering the viral agent (eg. Adenovirus or Lentivirus) for tumour induction is done under general anaesthetics. This is a non-invasive procedure where a small drop of viral agent is applied at both nostrils. Tumours - Tumour incidence varies with method of induction and genetic background. The majority of studies do not require advanced tumour development therefore 75% of tumour bearing animals will be killed by a Schedule 1 method before moderate adverse effects are exceeded. 25% of animals will harbour advanced tumours. General anaesthetic will be used but animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be killed by a Schedule 1 method.

- Mice might be injected with cancer cell lines to perform xenograft tumour growth assays. Cells mignt be injected subcutaneously or through the tail vein.

Post-surgical infection is unlikely (1%) as aseptic techniques are used. However, in case of an infection developing mice will be killed by a Schedule 1 method. General anaesthetic will be used but animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Any animals that fail to do so or exhibit signs of pain, distress or of significant ill health will be killed by a Schedule 1 method. Wild-type cells injected subcutaneously are not expected to develop tumours.

However, tumour cells or cells carrying novel cancer mutation may develop tumours.

At the end of all experiments mice will either be humanely killed, or tissues and organs collected under deep, terminal anaesthetic unconsciousness‘ or similar.

What are the expected impacts and/or adverse effects for the animals during your project?

To be able to study the early steps of tumour development we will need to breed mice that will develop solid tumours. We will focus on breast, ovarian, lung and digestive tract tumours. For some mouse lines they will be administered with non-toxic gene inducing agent via injection, water or food. In some cases mice will be treated with carcinogenic agents to induce tumour initiation. In all cases tumours will develop within several months. As most of our focus will be on the early steps of tumour initiation, we will have only a small proportion of our animals with fully developed tumours. In some cases, a potential therapeutic agent or preventative agent (eg. Vaccine) will be administered in cancer prone mice to determine their effect on tumour initiation.

General anaesthetic will be used for surgical procedures. Animals are expected to make a rapid and unremarkable recovery from the anaesthetic within two hours. Surgical procedures will be carried out aseptically. In the unlikely event of post-operative complications, animals will be killed unless, in the opinion of the Named Veterinary

Surgeon, such complications can be remedied promptly and successfully using no more than minor interventions. In the case of wound dehiscence, uninfected wounds may be re-closed on one occasion within 48 hours of the initial surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

Mild 40%
Moderate 60%

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cancer is a complex disease that develops in intact tissues. Studying primary human tumours at the molecular levels have been very informative as demonstrated by the international cancer genome consortium (ICGC) and the cancer genome atlas (TCGA) and ICGC studies. However, it is necessary to have a realistic model, which is amenable to genetic, and biochemical studies whilst maintaining tissue architecture. The mouse allows us to perform such detailed genetic and biochemical studies whilst maintaining the 3D tissue organisation and normal physiological environment. We will aim to use laboratory based cell lines whenever possible to perform validation and biochemical studies. However, cell lines have their limitations as they are a) self-selecting populations of cells that can survive on plastic and b) grow in a 2D environment. We will switch to animal experiments when the data at hand clearly supports the need for it.

Which non-animal alternatives did you consider for use in this project?

Cell lines and primary organoid cultures. Organoids are small, three-dimensional tissue cultures that can be grown in a dish in a lab into such an ordered pattern that they mimic a lot of the complexity of an organ and remove the need to repeatedly collect animal tissue.

Why were they not suitable?

The mouse allows us to perform such detailed genetic and biochemical studies whilst maintaining the 3D tissue organisation and normal physiological environment. We will aim

to use cell lines whenever possible to perform validation and biochemical studies. However, cell lines and organoids have their limitations as they are a) self-selecting populations of cells that can survive on plastic/matrigel and b) grow in a 2D environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We aim to identify the earliest cellular and molecular events that occur prior to epithelial tumour development. To achieve this we will use Genetically Modified (GM) animals which we have extensive experience in using. The experimental design overlaps considerably between the various epithelial tissues. Calculations were also informed by reference to the National Center for the replacement, refinement and reduction (NC3Rs) experimental design assistant. Mice will be randomly assigned to experimental or control groups whilst maintaining a comparable age, size and sex if possible (this is not feasible for breast cancer studies). The team of researchers will each be assigned a role to ensure that experimental and control groups remain blinded to the researcher designing the experiment. We have also performed this type of analysis previously and based on that published analysis we now have an informed estimate of the number of animals needed. In some instances pilot experiments might be needed to determine the number of mice necessary to achieve robust statistical results when experiments are ready to be conducted in full.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For new and pilot studies we will consult the NC3Rs experimental design assistant following the PREPARE guidelines. In addition, we consult with our bioinformatics collaborators ensure experiments are powered appropriately for downstream computational analysis.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most of our GM animals have complex genotypes we have carefully planned breeding strategies to maximize the number of suitable experimental animals and control littermates. All animals will be humanely killed at the end of experiments and tissue samples taken for further experiments. Where possible mouse tissues will be shared amongst the research group. Where appropriate pilot studies will be conducted to determine feasibility and efficacy. Depending on the tissue and scientific question, mice

will be randomly assigned to experimental or control groups. In addition, we try and run most experiments with at least two team members to enable blinding of the groups thus minimising bias.

Finally, when ever possible we always try and store and share tissues with the extended research community to maximise the amount of data and information collected from each mouse used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use the mouse to model and study the early steps of cancer development. We will generate genetically altered mouse models that represent the types of cancer seen in the clinic. We will breed these mice and monitor them for tumour development and tissues will be collected from these animals and analysed to identify the earliest signs of tumour development. Importantly, these models generate tumours with predictable latency and growth thus allowing termination of the experiment at an earlier stage when tumours are still relatively small. Thus, our tumour models mean that most mice are likely to exhibit only moderate adverse effects. The small number that exhibit severe effects will be killed immediately. We will use tissue specific genetic alterations which will minimise unnecessary harm for the animal and model the disease more accurately.

Why can't you use animals that are less sentient?

Cancer is a complicated disease that starts well before a tumour is detected. It is also a fact that cancer cells do not grow in isolation but do so by interacting with and manipulating the healthy cells in their surroundings, which is called the cancer micro-environment. By understanding, how cancer cells manipulate the micro-environment during the early stages of cancer hold the key to the development of new methods of early cancer detection and therapeutics. Therefore, it is not possible to use more immature stages or terminally anesthetised animals to answer this important and complex biological question. Mouse physiology is sufficiently similar to that of humans thus, enabling us to model human disease with a high degree of accuracy. This is facilitated by detailed knowledge and comparison of mouse physiology and genetics.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will aim to house the mice in social groups of up to 5 to enhance their social interactions and experience. If mice undergo surgical procedures we will administer pain medication and use post- operative bedding, heat mats if anesthesia was prolonged and provide mash for easy access to food. Animals are expected to make a rapid and unremarkable recovery from the anesthetic within two hours. In the uncommon event that animals fail to do so or exhibit signs of pain, distress or of significant ill health they will be killed by a Schedule 1 method unless a programme of enhanced monitoring and care is instituted until the animal fully recovers. Any animal not fully recovered from the surgical procedure within 24 hours (eating, drinking and return to normal behavior) will be killed by a Schedule 1 method.

Tumours - Tumour incidence varies with method of induction and genetic background. The majority of studies do not require advanced tumour development therefore 75% of tumour bearing animals will be killed by a Schedule 1 method before moderate adverse effects are exceeded. 25% of animals will harbor advanced tumours. Tumour burden will be measured using non-invasive methods such as Calipers. We will also explore the use of more advanced and quantitative non-invasive devices such as the Biovolume 2000 (https://www.biovolume.com/).

Pain management - After consultation with the veterinarian and if needed, pain management treatment could be provided in flavored jelly, paste or milk shake formats.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We adhere to the guidelines published in Workman et al (2010) Guidelines for the welfare and use of animals in cancer research. BJC 102, 1555 - 1577. We also consult the PREPARE guidelines when designing and planning animal experiments. It is also important that we report how animal experiments were conducted so that others can learn from our experiences and minimise potential animal suffering. For this we adhere to the NC3Rs ARRIVE guidelines on reporting of In Vivo Experiments. For surgical procedures we adhere to guidance from LASA such as the 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/current_publications/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are advised of advances in the 3Rs via regular email correspondence with our dedicated technician who regularly attend relevant 3Rs meetings. We also keep up to date through the use of the NC3Rs website pages (https://nc3rs.org.uk/resource-hubs) and 3Rs tools in-house and external resources such as Norecopa https://norecopa.no/databases-guidelines. In addition, we also regularly consult the Jackson Laboratories website which is

a valuable online resource that include the latest updates in animal husbandry and care. (https://resources.jax.org/)

155. Imaging neurons to understand memory formation and loss

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- o Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Optics, Brain, Memory, Synapse

Animal types	Life stages
Mice	adult, juvenile, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aims of this project comprise of two parts. The first will involve the development of novel, high- resolution, optical imaging instrumentation to conduct miminimally inavasive in vivo imaging in the mammalian brain. The second will apply the technology to address neurobiological questions, specifically the role of synapses in memory formation and loss, that would be difficult, if not impossible, to address in the absence of the new tools.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The fulfilment of the project aims will produce two important bodies of work. The instrumentation we build has a direct impact in the area of human neurosurgery. At present we are funded to develop a prototype of a minimally invasive device for brain surgery. Critically, the applications for our technology do not solely rest with brain imaging, they are likely to be suitable for any live tissue work where access is challenging, but minimising tissue damage is a priority.

The second area where we will make an impact is the application of the new technology for the study of mammalian synapses. The loss, gain and the structural rearrangement of synapses is observed in almost all neurological conditions including Alzheimer's Disease, schizophrenia and autism. Each of these diseases come with a huge societal burden. For example, current published data report that there are nearly 6 million Alzheimer's sufferers within the USA with the number projected to rise to 13.6 million by 2050. Thus the need to better understand what drives the changes at the synapse could barely be greater.

By imaging synapses in vivo, we are confident that these crucial structures are examined in the correct biological context. Thereby the scientific observations we make can be quickly and appropriately applied to the normal or pathological status of synapses in humans.

What outputs do you think you will see at the end of this project?

The outputs will take a number of forms. First we will publish our work in peer reviewed journals. This will represent the primary mode for dissemination of new information. Second, we will patent or licence relevant material when appropriate do so. This will be most relevant for the optics developments. Most technological developments, including software, will be quickly released to the scientific community, with ready access to the materials and techniques via a website that we support.

Who or what will benefit from these outputs, and how?

This work will develop optical technologies that will allow high-resolution image collection to be performed not only within superficial regions of the mammalian brain but also areas deep within it. These advances stand to afford substantial benefit to a large community of researchers and clinicians.

Many neurological conditions have their origins in brain areas that are inaccessible to high-resolution brain imaging. Examples include Alzheimer's Disease, where synapse loss within the hippocampus, a deep brain structure, is thought to be an early indicator of disease onset. Other deep brain areas, such as the nucleus accumbens, are linked to the changes that accompany the onset of addiction, a huge societal problem, and yet study of these hugely important brain regions is hampered by the lack of tools that would permit optical interrogation of their neurons and synapses in vivo. The development of the optical tools within this project will therefore extend benefit to a wide community of researchers whose work will be enhanced by new instrumentation of this type.

As part of the project we will also make our own contribution to the study of synapses with a view to better understanding changes that take place as the mammalian brain learns new information and critically the reverse, the changes that occur as information is lost. Information loss is particularly important as there are occassions where it is desirable and necessary to forget and occassions where forgetting represents a serious health concern, such as with dementia. An understanding of these biological processes is a crucial step towards the development of diagnostics and interventions that can serve to ameliorate or perhaps cure such pernicious diseases.

Finally, the value of the instrumentation extends beyond pure research tools, with the significance for human diagnostic having already been recognised. We are presently fully engaged with the neurosurgery community and building an instrumentation for use in humans.

How will you look to maximise the outputs of this work?

As a group we collaborate extensively with experts in the field of adaptive optics, MMF technology, statistics, neurosurgery, and synaptic plasticity. We share with these groups our progress and this information exchange is reciprocated. We would not hesitate to seek further assistance and collaborators should we require support in a specific area, nor will we restrict assistance to those who wish to share in our experience.

As an illustration of the extent to which we actively seek to share our developments we are presently seeking grant support (Wellcome Trust) to offer workshops where other scientists can visit the laboratory to learn about implementation of the techniques we are developing, this support would also secure development of on-line resources.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used as this is an animal capable of robust, demonstrable learning. It’s brain architecture is similar to that seen in humans, this includes the presence of dendritic spines at excitatory synapses.

Dendritic spines are known to be of importance in human neural pathology and are the first structures to change in memory related diseases. Our primary interest is in normal, mature synapse function and so we will study healthy adult animals, in this way we will not conflate our results by those that reflect developmental or disease processes.

Typically, what will be done to an animal used in your project?

The ambition is to image neurones and their synapses deep within the mammalian brain for the purpose of understanding the changes that occur at synapses during memory formation and memory loss. In order to gain optical access to the brain animals will undergo a single surgical procedure to permit either the insertion an optical fibre or fibres, or to remove a small piece of the skull to be replaced with a transparent cranial window. All surgical procedures will be conducted whilst the animal is under general anaesthetic. Once fully recovered from the impact of surgery and anaesthesia animals will be undergo cycles of imaging for periods not exceeding 3 hours, where typically structural, and sometimes functional, information will be gathered. Whether the animal is anaesthetised during these imaging cycles will vary. For animals for whom the head is to be held a light plane general anaesthesia will be used. However, for some animals the imaging technology will have reached a point in its development to be sufficiently refined as not to require restraint of the animal nor anaesthetic. In all cases imaging can take place across several weeks. The interval between each round of imaging may be as short as a single day, although the interval may also be longer. The interval between imaging cycles will be determined both by the biological question and the level of impact that an imaging session has upon an animal. Imaging in most animals will occur without intervening interventions, such as administration of substances, although some animals may receive a substance or be sampled (blood or swab) in order to measure the level of a hormone. For some animals behavioural training between imaging sessions may also occur with a view to understanding what impact this has upon synapse form and function. At the end of the image collection cycles all animals will be humanely killed.

What are the expected impacts and/or adverse effects for the animals during your project?

All animals will undergo a surgical procedure under general anaesthetic. While the impact of these procedures is expected to be modest it is recognised that the animal may experience some post- operative impact that could include, pain, inflammation, blood loss, or a period of weight loss induced by the anaesthetic. Each of these could potentially drive a change in the animal's normal behaviour. The impacts described are expected to be short in duration, i.e. will not extend for more than few days.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

50% of mice will be subthreshold.
50% of mice will experience moderate severity.
What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Learning and memory are processes that are driven by the behaviour of the animal. In order that we may make accurate attributions to neural function in relation to changes in the behaviour we must be able to relate brain activity to behaviour. Thus we look to study brain function in animal models where we can simultaneoulsy measure brain activity and behaviour. The optical technologies that we will develop and refine may ultimately be suitable for use in humans. The size of the human brain is very large relative to our instrumentation ensuring that the invasive impact of the device is minimal (negligible). However, assessment of its utility requires that we first examine the tool(s) in the living mammalian brain tissue of animals, thereby helping ensure that our technology is fit for purpose.

Which non-animal alternatives did you consider for use in this project?

All of the early stages of the development of our optical tools are conducted without the need for animals, or indeed biological tissue of any type. The optical performance of a device can be assessed by physical means and only once the device adequately fulfils stringent optical requirements do we consider implementing the technology in the context of tissue imaging. This stage begins with cadaveric material and studies of ex vivo tissue and only once these are successful do we commence work in vivo.

Why were they not suitable?

Model systems are not suitable for the final stage in our studies for a number of reasons. The first is that the structures we wish to understand, synapses, are subject to many competeing physiological parameters, many of which will exert an influence upon the biology of the synapse. One example would be the cyclic presentation of hormones. Something, that is hard to artifically recreate. The second relates to the type of questions we wish to ask, specificlly about the performance of synapses in the formtion and loss of memory. To achieve this demonstrable learning, recall and forgetting will need to be demonstated, something that can only be determined in a behaving animal.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have been conducting related experiments under licence. The new licence looks to develop this program of work. We are therefore familiar with the number of animals required to support this type of work as well as the numbers used in direct imaging experimentation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will implement a staged process in our investigations that ensures of use of animals is kept to a minimum. Step one assesses the optical performance of instrumentation without the need for animals. Key optical performance parameters will need to achieved prior to moving to step two. In this step we examine the performance of the technology in ex vivo tissue taken from animals under schedule 1.

Success at step two then will allow us to move to step three where we test the technology in vivo in animals under terminal anaesthesia. Only if successful at step three will we move to move to step four, the neuroscience experimentation component of our studies. Here again we follow a staged process, with combinations of options chosen to maximise the amount of information collected from each animal.

As a fundamental goal of our optical technology is to permit repeated measurements to taken from a single animal we radically reduce animal numbers by the nature of our experimental design. Data are collected from single animals before and after learning, therefore we always have a within animal control. In this way the need for examination of large populations of animals to ‘smooth’ biological noise is much reduced. A further constraint, that helps reduce animal numbers relates to the complexity of the optical hardware, we build bespoke optical devices and so typically have only a single device for expeimental use. This consideration, combined with surgeries, places a very real brake on the number of experiments we can perform. The measurements we take are usually simple to interpret, requiring minimal statistical power. In essence images are compared and assessed for quality against those those produced from ex vivo tissue. The observations we wish to make following behavioural training will seek to identify macroscopic changes in synapse number, size or transmission performance and also require relatively modest statistical power as the baseline condition represents data from the same neuron(s) prior to training.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The care and maintenance of our animal breeding colonies is monitored by expert NACWOs and animal facility staff. Excellence in animal husbandry techniques help ensure that the number of animals we breed aligns with best practice for sustaining a healthy colony.

Almost all instrumentation development commences with the generation of computer models to assess the design and likely success of the imaging tool. Devices are then built and then, as discussed in the previous section, images are taken first of calibarated beads and then with ex vivo tissue. Only once these images are fully validated is any attempt made to move to in vivo imaging.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We wish to use mice and take advantage of the vast array of genetic tools available for mice, in particular transgenic lines expressing genetically-encoded fluorescent reporter proteins in defined neuronal population. The optical methods we use to interogate neuronal function are designed to be minimally invasive, this is desirable from an experimental refinement perspective as this approach is least likely to peturb the biology, but critically in this context the size of our optical fibres, or the optical access techniques to be applied will help ensure that minimal physical damage to the animal occurs and in consequence the least pain and distress.

Why can't you use animals that are less sentient?

We describe how animals that have been terminally anaesthetised will form a crucial part in our study. However, a key objective within the work requires that animals have undergone demonstrable learning and exhibit memory. These biological phenotypes require that the animals are sentient in order that these behaviourally driven qualities may be observed. While it is true that less sentient species are capable of learning and memory, whether the neural mechanisms involved are comparable with those seen in humans is contentious. What is less contentious is that the capacity for sophisticted learning decreases with decreased phylogentic sophistication. We have therefore chosen a species that shares many parallels in brain structure and neural performance with humans. The life stage chosen for our studies are adult animals. We judge this appropriate as immature animals are subject to synaptic plasticity, a normal part of their development. We wish to be sure that we do not conflate changes in synapse number, type or function

that arise in development with an adult process, as it is the adult synaptic changes that we believe to be most relevent to human diseases we seek to understand.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Surgical interventions:

The hallmark of our experimental protocols will be the use of optical fibres or cranial windows to achieve chronic cellular and sub-cellular imaging of neurons. Previously, others have reported imaging of deep brain structures like the hippocampus after surgical aspiration of the overlying cerebral cortex. Our approach is fundamentally different in that we aim to cause as little damage as possible to the brain and will use probes that can be as small in diameter as a human hair or optical methods that obviate the need for aspiration. For example, the diameter of the MMF device permits direct insertion into the brain and generates so little damage that imaging can be performed almost immediately thereafter.

We will consult closely with the university veterinary services to improve and refine surgical protocols. Any significant refinement in our methods will be initially tested in dead animals (killed by schedule 1 methods) or under non-recovery anaesthesia. We will be using state-of-the-art surgical equipment and designated rooms, with optimal aseptic standards and animals are administered post-surgical analgesia. Animals in our study express fluorescent reportors removing the need for the injection viral or fluorescent agents.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We receive all updates for NC3Rs – National Centre for the Replacement, Refinement and Reduction of Animals in Research. https://nc3rs.org.uk These include webinars, links to specific relevant publications e.g. Minimising mouse aggression, Sci reports (Oct 2019) as well as principle and practice videos.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As part of our Establishment's Gold Standard practice all licence holders must attend termly animal welfare meetings. These groupings are organised to map onto local AWERBs, ensuring that participants share common research practices. A specific agenda item for these meetings explores advances in the 3Rs, ideas are exchanged and best practice discussed. Three crucial communities also present at these meetings are the vets, NACWOS and the Regional Programme Manager NC3Rs

– National Centre for the Replacement, Refinement and Reduction of Animals in Research, all of whom provide updates and advice for implementation of any advance.

156. The biology of body weight and body composition

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Obesity, Cachexia, Body composition, Diabetes

Animal types	Life stages
Mice	adult, pregnant, juvenile, embryo, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how body weight and body composition are controlled and how disturbances in this control lead to disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Understanding the processes that govern body weight is highly relevant to clinical practice as disorders that arise when these processes go wrong cause a great deal of illness and suffering. Obesity, defined as excessive storage of energy as fat, is a serious issue that drives medical conditions, with obese individuals at increased risk of developing problems with their heart, blood vessels, kidney and liver, as well as being more likely to be affected by a number of different cancers. These problems bring added personal burden to the

affected individual and the healthcare support needed to treat these diseases costs a lot of money. In 2017 Public Health England reported that the UK-wide NHS costs attributable to overweight and obesity would reach £9.7 billion by 2050.

At the other extreme, cachexia is a syndrome of negative energy balance where muscle and fat mass are progressively lost. It affects over a third of all cancer patients and is strongly associated with both reduced tolerance to anti-cancer therapy and reduced survival times.

What outputs do you think you will see at the end of this project?

In undertaking these studies, we will generate new **data on the biological process that control body composition. These data will combine with our on-going studies in human populations and will be presented in peer-reviewed publications and shared with colleagues in academia, medical sciences and industry.**

Who or what will benefit from these outputs, and how?

In the short term the major beneficiaries will be the metabolic scientific community. We also expect our research to be of benefit to the fields of cancer biology and cardiovascular disease.

In the longer term we expect our research to be of benefit to the pharmaceutical industry, where the genes and processes we identify will provide new targets for the creation of drugs to combat body weight-associated diseases with the ultimate goal that will, even in a small way, improve human health.

Finally, we believe that work under this licence will have a role in shaping and training the next generation of researchers working on understanding how "whole body physiology", the important science of understanding how each of the multiple component parts of the body communicate to each other to allow the body as a whole to survive and thrive.

How will you look to maximise the outputs of this work?

We will share our data and ideas with national and international collaborators and will publish our work in open access publications. We will look to share all the data we generate. We believe that it is as important to share outputs and insights that show a pathway or molecule does not have a key role just as much as it is to highlight significant insights from more novel results. We also have an active engagement with the public via lay science pieces, national newspapers and Twitter.

Species and numbers of animals expected to be used

- Mice: 17,200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Human metabolic disease typically comes about because of the complex cross talk and interaction between external factors (like diet, activity and environmental conditions) and internal factors such as levels of hormones in the blood and chemical signals in the brain. We believe that mice have the necessary, very similar organ structure and hormonal systems to humans that means studying them is going to be a really informative way to improving understanding of the "chemicals of life" like sugar and fat that are knocked out of balance in human metabolic diseases like obesity and diabetes.

Mice also offer a system in which precise changes in genes and proteins of interest can be made to really focus in on the detail of how the component parts of a mechanism all link in and work together.

As the majority of human metabolic disease affects adults, most of our work will be in adult mice. However, we recognise that these diseases only emerge after many years of exposure to particular diets and lifestyles so to be able to replicate this in a model system we will need to study younger animals.

Typically, what will be done to an animal used in your project?

The majority of the work will focus on studying animals that have been designed to either make a gene not work at all (“loss of function”) or work at a higher rate of activity (“gain of function”). We particularly want to know how these changes in gene function alter the responses to environmental challenges and drugs used to treat disorders of body compositions.

These procedures can be broadly grouped into 3

Procedures needed to create the model; the genetic engineering done in the laboratory sometimes need additional delivery of other compounds to complete the process. Whenever possible these reagents will be given by the oral route (either by mixing in the diet or by a tube inserted via the mouth into the stomach) but sometime may need to be delivered directly into the body by injection. These injections may be intravenous (into a vein), intraperitoneal (into the body cavity) or subcutaneous (under the skin). As we are focused on body weight, we will frequently change the dietary intake of an animal, both in terms of constituents and amount consumed. This can mean supplying a diet that will make the animal put on weight as well as restricting food to bring about weight loss.

- Challenges to test the system; animals will be given drugs, naturally occurring hormones and biological active reagents such as antibodies. These will mostly be delivered by injections into a vein, into a body cavity or under the skin but sometimes may require the placement under the skin of small pellet-like, devices that act as a depot for drug delivery over several weeks. Because we understand that the brain has such a crucial role in controlling body weight, we will sometime need to undertake surgery to enable us to deliver drugs and hormones directly into regions of the brain that we know control how we eat or expend energy. When we do this the mouse will be under a general anaesthetic and will be unconscious. A mouse will only usually have this kind of surgery once and the procedure takes around 20 minutes. We use a specially designed operating table that enables small sterile tubes to be placed into specific regions of the brain accurately and quickly and the mouse is usually fully recovered and back to eating and drinking within 2 hours.

- Processes needed to measure and analyse the response; animals will be placed in carefully designed study chambers and imaging scanners to measure their behaviour and response to these challenges. These special study chambers look very much like the houses the animals normally live in through the week but with some simple modifications to enable, for example, sampling and measurement of the oxygen levels in the air circulating in the chamber or measurement of how much food and water the mouse has consumed. As such, the mice can easily spend several days in these chambers without undue stress. Similarly, when the mouse needs to have a scan to check how much fat and lean tissue they have, they will be placed in specially designed tubes made of material identical to that found in the home cage to enable them to remain still and secure and quickly have a scan without the need for sedating drugs. Finally, these response measurements will sometime involve taking small amount of blood from superficial veins.

A typical experiment will involve a small series (2 or 3) of short interventions over 3-4 weeks. For example, we will give an animal a highly palatable diet for 2 weeks, treat it with a drug for a week and see what effect that had on the food intake, the body weight and the energy expenditure of the animal.

What are the expected impacts and/or adverse effects for the animals during your project?

Most experiments proposed will lead to no more discomfort than that experienced by any rodent bred in captivity and residing in a modern animal facility.

Some animals will experience transient (seconds) discomfort when given injections or when having blood samples taken. The injections will often be of naturally occurring hormones, or compounds closely related to them. On occasion, animals may be given compounds that are recognised to produce circulating levels of hormones that are seen in acute illness. These may reduce the animals' drive to seek out and eat food in the hours after they have been given.

When given a different diet or treatment, some animals will gain or lose weight. This will be within closely monitored parameters that take into account other important aspects of their appearance and behaviour. This weight change will typically occur slowly over weeks.

A minority of animal will also undergo surgery that will require a general anaesthetic. Inevitably, as with any operation, animals will have some discomfort in the immediate hours after the operation at the site of the incision. However, this will be minimised by administration of painkillers under instruction from a veterinarian. The general anaesthetic needed for this surgery may also make the animals less active and less hungry in the first day after the operation but we expect them to recover their appetite and vitality within 48 hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Breeding protocol 95% Sub-threshold, 5% mild
- Other protocols
- Mild- 65%
- Moderate-35%
- Severe- 0%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Human metabolic disease is the end-result of a complex interaction between multiple external environmental factors and internal hormonal, chemical and neuronal messengers. This cannot be meaningfully replicated in anything other than animal models and although we could use non- vertebrate animals which are of lesser sentience than rodents to help in our studies, none have the necessary complexity in organ structure or wider networks to adequately address the scientific questions posed. Further, the need for targeted genetic sophistication and the need to access deep internal tissue such as a brain requires a model organism system.

Which non-animal alternatives did you consider for use in this project?

We look to use animal alternatives where possible. We use cell culture models for looking at specific mechanisms that occur in isolated cell populations. Results from these experiments can both be informed by our animal work and help us to design better animal experiments.

When looking to ask a question about the function of a gene found in the brain we have used neurons grown in a cell culture medium rather than use mouse brains. For example, through our links with colleagues who study human genetics, we have identified a number of possible genes that are linked to obesity. We also know from published work that these genes are expressed in the brain. These make them exciting candidates for future projects but before we do any work in mice, we want to be really sure they are working in the way we predict. To do that we have altered the function of these genes in neurons grown in a dish to see what happens, replacing the need to do these preliminary studies in animals Further we have collaborated with colleagues who have gut “organoids” to test ideas on hormone action in the gut. Organoids are small, three-dimensional tissue cultures that can be grown in a dish in a lab into such an ordered pattern that they mimic a lot of the complexity of an organ, and remove the need to repeatedly collect animal tissue. Finally, with correct ethical approval and process in place, we have access to post-mortem material from humans to enable us to map out the location of genes of interest without using animals at all.
Why were they not suitable?

While cells and organoids grown in a dish are useful they can never generate the **integrated data essential to this project. We cannot record from a nerve cell how fat it has become or if it is hungry, or determine how much an organoid has eaten. As such they not a sophisticated enough model platform to enable us to study the complex interplay between multiple organs that leads to metabolic disease.**

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.
How have you estimated the numbers of animals you will use?

These numbers are largely based on the level of work over the previous two licenses and the amount of funding we have in place and expect to use to fulfil our aims and objectives.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To avoid wastage of animals, appropriate background research will be done prior to all experiments. Whenever possible, we will look to work with existing colonies of animals rather than breed new colonies. We will allow other trained researchers to work with the colonies in our unit rather than moving mice, reducing the number of mice that are both bred and transported.

Studies will be of appropriate size to detect significance, with animals randomly assigned to matched study groups and, whenever possible and practicable, investigators blinded to the nature of interventions. Protocols will include a series of analyses and steps on a single animal, rather than single analyses on multiple animals. We aim to balance impact upon an individual animal with scientific output but reason that this approach significantly reduces the number of animals used.

We think carefully about which other organs researchers in the laboratory can work on, such as heart and adipose tissue, and harvest these from the same animals. This reduces the numbers of animals used overall, increases the amount of data obtained from a single animal and allows us to examine links between different tissues/organs by combining data from the same animals, thereby enhancing the quality of the information produced.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We use pilot studies for any new agent we are investigating. These are used to check the safety of drugs in a small number of animals before using them in a larger cohort but also give a real world output on effect size to design the larger study.

We routinely freeze down and store either eggs or sperm from all of the different colonies of mice we study. This means we only breed animals when we need them for experiments, rather than having to maintain a permanent colony for use in potential future experiments. We collaborate with other groups across the UK, Europe and the USA and share samples we have banked to these collaborators for their own purposes, maximising the benefit from previously conducted studies.

We are also aware that we live in an age when more and more experimental data are placed in open access platforms that are easily accessible on-line. We continue to work closely with our experts in information technology to look through these large data sets to find information that will actively shape our experimental design.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.
Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use mice.

We will typically use a mixture of wild-type, genetically altered and naturally occurring genetic mutant animals. Genetically altered animals are those that are carrying a specific and deliberate change in their genetic code that has been engineered to be **there through laboratory manipulation of DNA.**

We will use altered diets to change body weight. These are formulated to be accessible and palatable to mice.

In terms of the tests we carry out to measure circulating chemicals, either in the resting background state or after the system has been "switched on" with a challenge, we have refined protocols which deliver the smallest volume and require the smallest sample sizes possible to generate meaningful data.

Imaging and calorimetry systems are widely used apparatus that have been continually refined and redesigned over years to minimise stress. Calorimetry systems are cages plumbed in to closed circuit gas analyser systems which enable measurement of what gas a mouse had consumed and what gas a mouse has produced. In doing so, this enables a calculation to be made of how much energy that mouse has used up. While previous calorimetry systems had rather barren grid floors, continuing refinement in design and material has now made it possible to have a calorimetry system based around the existing base floor unit of the home cage.

To more accurately record food intake and metabolism, we will sometimes need to single-house mice. This can be stressful but we will minimise the time each animal is housed alone and, where possible, animals will be re-housed in groups with their original cage-mates following a period of single-housing. In addition to shelters, nest boxes and nesting material, tubes to act as hiding tunnels, shredding toys and wooden chewing toys for animals to gnaw upon will also be supplied. These will be modified as necessary to accommodate head placed cannula in any animal that has undergone surgery and had an indwelling cannula sited.

Finally, we have a number of specialised techniques that we will perform at the end of the experiment on terminally anaesthetised animals to minimise their pain suffering and distress. Animals that are under "terminal anaesthesia" remain in a state of deep sleep and unconsciousness throughout until they are humanely killed at the end of the experiment.

Why can’t you use animals that are less sentient?

We use less sentient animals where possible and our research group has used flies to study specific research questions.

However, as our interest is in modelling human disease states, a mammalian system is the most suitable.

We do use terminally anaesthetised animals for some procedures, but under such conditions we cannot measure processes such as food intake and energy expenditure that can only be measured in live animals over days.

How will you refine the produres you're using to minimise the welfare costs (harms) for the animals?

We have made several refinements to our techniques over the past years. Specifically; More prolonged period of acclimatisation in an enriched environment with “same-user handling” in period prior to studies where stress is anticipated and where stress will affect data output. This has been particularly useful in studies of the stress hormone corticosterone where, without a period beforehand where the animals are used to being handled, the stress signal from the test itself may have swamped the true biological signal. Whenever possible, mice will be housed in social groups.

For calorimetry data we have taken advantage of in-house, mathematics expertise existing within our institute to factor in the inevitable change in body weight seen in the study period. This improves the quality of the data and removes the need for the animals to undergo a repeat period in the calorimetry system.

For food intake studies we have made our own dishes to capture spillage and made measurement of food intake a much faster and accurate process, maximising data yield from the time animals spend single-housed. Whenever we are using any drug that we wish the animal to take by mouth, we will work with our teams in the animal units to introduce appealing and attractive flavouring (like Strawberry Nesquick) to be delivered alongside the drug.

We will look to use close observation of both body weight and body condition scoring to get a more complete readout of an animal's situation to enable us to detect problems early and avoid harm. The body condition scoring system is a simple, rapid and noninvasive method for assessing health status and wellbeing.

In any post-operative period we will pay close attention to ensuring the environment meets the needs of the animal by the addition of a heated environment, post-operative bedding and more palatable mashed food.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.lasa.co.uk/current_publications/)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning animal research and testing (https://www.ncbi.nlm.nih.gov/pubmed/28771074)- used for planning our experiments
Smith et al. Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Animal 2018 Feb;52(1_suppl):5-57. doi: 10.1177/0023677217744587.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our institution has a regular advisory board meeting to facilitate meaningful dialogue across the license holder, researchers and technicians. Through these meetings we are able to find out about latest practical guidance from relevant national bodies such as the LASA (Laboratory Animal Science Association) and the RSPCA (Royal Society for the Prevention of Cruelty to Animals).

Accessing NC3R website (https://www.nc3rs.org.uk) as a resource for guidelines, practical information, links to publications and training materials.

Advances in the 3Rs will be disseminated to those operating under this licence through the weekly laboratory meetings.

157. Repair and protection of the retina and optic nerve

Project duration

5 years 0 months

Project purpose

- Basic research
  - Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Blindness, CNS disease, glaucoma, nerve regeneration, neuroprotection

Animal types	Life stages
Mice	adult
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To test new treatments for enhancing protective and regenerative machinery in the optic nerve. These treatments aim to treat loss of vision due to glaucoma, and to regenerate fibres in the injured or diseased central nervous system.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The aim of this work is to treat disease or injury to nerve cells (neurons) in the optic nerve and retina. The intention is to develop new treatments for the degenerative eye disease, glaucoma. Additionally, the findings will be relevant to treating many neuronal diseases or injuries in the brain or spinal cord.

Glaucoma is one of the leading causes of blindness worldwide. Glaucoma is a neuro-degenerative disease which causes the nerve fibres (axons) between the retina and brain to deteriorate. This ultimately leads to the death of the nerve cells (neurons) in the retina which extend their axons towards the brain. Currently treatments to protect retinal neurons are not very good, and there are no treatments that allow regrowth of damaged axons back towards the brain. Adult retinal neurons are similar to neurons in the brain or spinal cord, in that they do not regrow after they are damaged by injury or disease.

The aims of this work are to identify and test new treatments that will (1) prevent retinal neurons from dying during glaucoma or after other injury or disease to the optic nerve, (2) enhance the regrowth of damaged fibres. Our work has shown that axons in the optic nerve, brain and spinal cord do not regrow after injury or disease because the cellular machinery for protection and regeneration is not abundantly there. We have discovered some treatments that allow this cellular machinery (components that trigger growth or survival) to be directed into the nerve fibres. This enhances the ability of nerve fibres to regrow, and prevents retinal neurons from dying. We have tested many treatments using models of injury and disease in cell-culture dishes, reducing the need for animal experiments, but ultimately, we need to test these treatments using animal models of retinal and optic nerve injury or disease, so that we can see whether they are worth developing to treat patients.

We also need to fully examine specific protective and regenerative molecules (cell components) into the optic nerve. We know about some of these molecules, but we need to understand associated processes much better in order to identify better treatments.

What outputs do you think you will see at the end of this project?

As with our previous work in this area, we will publish our results in high-impact peer-reviewed journals. Our latest manuscripts were published in broad-appeal and clinically relevant journals, meaning the work is read not only by clinicians interested in potential new therapies but also by scientists from many different disciplines interested in scientific advance.

We would also aim to identify potential treatments that are novel and unexpected and suitable for business development. Suitable therapies with high potential for clinical translation will be considered for patent applications.

Who or what will benefit from these outputs, and how?

Our research appeals to scientists investigating novel treatments for glaucoma, and repair of the injured or diseased retina and optic nerve. Clinicians in this field are also very

interested in our work, keenly anticipating potential new therapies. The work is also of interest to the central nervous system (CNS) repair community. Interventions which enable axon regrowth in the optic nerve are usually efficacious in the brain and spinal cord, so researchers looking to repair the injured spinal cord are also very interested in our findings.

Ultimately, our research is aimed at developing new treatments for eye disease. The intention is for our work to benefit the vast number of patients worldwide who are suffering from loss of vision due to degenerative disease in the retina and optic nerve.

The work will also be widely disseminated to the scientific community through publications and the findings will be presented at local, national and international scientific meetings.

The data will also be used in support of future funding applications and potentially for patent applications for development towards clinical treatments and enterprise.

How will you look to maximise the outputs of this work?

We collaborate with many scientists throughout the world and build on our findings through further experiments which are better performed by these additional experts. Our recent papers were published together with many labs worldwide, and we are part of global research consortia. In addition to publication in high-impact journals, the work is also presented at conferences worldwide, within the eye research community, CNS repair community, and at specialist cell biology meetings aimed at understanding relevant cell biological mechanisms. Papers are usually published as open access articles, and can include positive and negative (unsuccessful) findings.

Species and numbers of animals expected to be used

- Mice: 900
- Rats: 550

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will use mice and rats, which have not been genetically modified. The group has extensive experience with both species and all new ocular drugs require testing in a mammalian system..

Mice and rats are used because their eye anatomy is similar to that of the human eye, including the response to injury. We use two models of optic injury and disease. The injury model is well characterized in mice, as is the disease model in rats. Rats are required for the disease model, because the larger globe size is necessary to accurately model

disease pathology. It is also useful to show that our novel protection and regeneration strategies work in more than one species.

These models need to be investigated in adult animals, because the response to injury is very different in young animals, and the disease we are modelling does not occur in young patients. The young central nervous system has a limited ability to regenerate after injury, whilst the adult central nervous system has almost none. It is the injured or diseased adult central nervous system that we are aiming to repair.

Typically, what will be done to an animal used in your project?

We have spent many years investigating the mechanisms that control the ability of nerves to repair after injury or disease, using cell culture models of injury, and we continue to generate new targets for repair using these models, which we make into new therapies. The animals used in our projects will typically receive an injection into the eye. This is a not painful, and is routinely used for many current therapies to treat patients with glaucoma and other eye diseases. The injection is intravitreal, which is a shallow injection through the white of the eye, to deliver a small amount of liquid into the vitreal humour inside the eye.

Animals are then left for a few weeks to allow for the treatment to work in the eye. The animal will then receive either an optic nerve crush (to model injury) or a laser injury to the eye, which results in raised eye pressure, mimicking disease. The optic nerve crush is performed under anesthesia, and involves moving the eyeball forward of the socket to expose the nerve behind. The nerve is crushed with fine forceps, and the eye returned to its usual position. The nerve does not sense pain, so the crush is not painful afterwards. Animals are left for some time (typically two to six weeks) for regeneration to occur. The animals are then killed, and we analyse regeneration in the optic nerve, and protection from cell death in the retina.

For the disease model, the animal is anesthetised, and a laser is used to cause a very small injury (similar to cauterising) to a specific area around the edge of the eye. This slows down the normal drainage of eye fluid, leading to a build-up of eye pressure, (similar to glaucoma). The injury heals, meaning that the pressure build up is transient, so after a week the injury is repeated. 42 days after the initial injury, the animals are killed, and the optic nerve and retina are analysed for degeneration,

regeneration, and protection from cell death. During the course of this disease model, eye pressure is measured regularly with a tonometer (which is like a blunt pen that is gently pressed against the eye), and eye function is measured by electroretinography ERG, a non-invasive assessment of retinal function using light and electrodes placed on the eye.

For ERG recordings an electrode is placed in contact with the surface of each eye, wire loops placed around the circumference of the eyes and an electrode placed into the tail or skin of the animal. This is done under anesthesia. During the same anesthetic, imaging

may be done, using a camera to image through the cornea to observe the retina at the back of the eye.

Tonometer measurements involve touching the cornea with a small probe which provides an instant resistance measurement based on the eyes intraocular pressure. This does not require an anesthetic.

What are the expected impacts and/or adverse effects for the animals during your project?

Neither the injury procedure nor the disease model are expected to cause adverse effects. The injections into the eye should not cause any complication, and the in life assessments are not painful and do not cause adverse effects. The group has several years' experience with these techniques and rarely encounter problems.

The eye procedures can cause temporary swelling and tenderness, but this should rapidly resolve itself. The animals will be very closely monitored after the procedures to ensure these effects subside, and if necessary, animals will be treated for pain or inflammation. Vision loss occurs rapidly after the optic nerve injury, and more slowly in the disease model. Loss of vision is limited to one eye, so the animals do not become blind from the procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

C57BL/6 (a wildtype mouse strain) – mild (50%) moderate (50%).

Wistar, Sprague-Dawley, Lister Hooded (wildtype rat strains) – moderate (100%).

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We have worked for many years using cell culture models to study the molecules that control the regenerative capacity of the central nervous system (CNS: brain, spinal cord and optic nerve). We continue to use these systems, which have proven to be incredibly valuable for identifying new targets for stimulating regeneration after injury or disease, as

well as protecting injured or diseased neurons from death. Our in vitro studies have discovered treatments that are effective at preventing cell death in the retina as a result of injury, and that they are very effective at stimulating regeneration of the fibres that connect through the optic nerve to the brain.

Whilst we have been very efficient at modelling CNS injury, the eye and optic nerve cannot be modelled in cell culture systems. In order to test whether our new gene therapy tools could be useful for treating patients, we need to find out if they can protect and repair in intact eyes and optic nerves. It is therefore necessary to investigate our new treatments using animals.

Which non-animal alternatives did you consider for use in this project?

We use cortical neuron cultures to model CNS neurons, and we are developing new human neuronal culture systems using inducible stem cell derived neurons. We also plan to pursue other cell culture injury models, including scratch assays, where we use a needle to injure a "hedge" of axons and monitor regrowth afterwards.

These are all well respected models of CNS injury. To model eye injury and disease we are working with collaborators who are experts in the retinal explant model.

Our philosophy remains to conduct cell culture experiments to identify new treatments, and to only use animal models for confirmation of therapeutic benefits.

We additionally considered culturing retinal ganglion cells (RGCs) in dishes, but these are notoriously difficult to culture, and they do not behave similarly to RGC neurons in a real-life situation (in live animals).

Why were they not suitable?

Whilst we use cell culture models to study mechanisms of nerve injury and neuroprotection, these models are not sufficient for this project because the cells we are targeting are influenced by many conditions which are not represented in cell culture. For example, the retina and optic nerve contain many cell types in addition to neurons, including immune cells, and to investigate whether our new treatments are beneficial in a meaningful fashion, we need to understand the regenerative or neuroprotective effects in a complete biological situation, where the RGCs are also influenced by the many other cell types. None of the available cell culture models completely model the situation in the injured or diseased eye or optic nerve, but we will continue to use cell culture systems to model aspects of injury or neuroprotection as closely as possible, and all off the new treatments we test have been previously studied in cell culture models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample sizes are calculated from previous similar experiments and from pilot data to minimise group sizes required to demonstrate clinically relevant effects. Pilot studies are always carried out using a small cohort of animals (3-6 animals) before beginning a full study.

Testing of preliminary novel treatments are typically performed on group sizes of 3 to 4 animals due to the reproducibility of the procedure and results. Controls are needed in order to correctly analyses effect size. In the case of our novel therapies, these usually mock delivery systems.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

For each of the procedures, there is a lot of prior experience within the group, and statistical advice has been obtained from the biostatistics unit. Experiments are conducted to the PREPARE guidelines, and will comply with the ARRIVE guidelines for publication.

Sham surgeries have previously been used to control for unexpected inflammation which may cause an unpredictable response, and are not required for acceptance as a published article. We are quite confident our surgeries only have the desired effect and are keen not to use excessive animals where possible to comply with the 3R's.

Any new surgery, and any new Procedure Individual Licence (PIL) holder will undertake preliminary experiments to generate data relevant to the technique and research question. Additionally, doses will be calculated from effective non-toxic concentrations in tissue culture studies to minimise dose ranges needed to be testing in animals.

The minimum number of control groups will be used for each experiment in order to reduce numbers whilst allowing for the experimental data to be statistically meaningful. For new treatments, we know from previous studies that phosphate-buffered saline (PBS) injection and mock delivery injection can be used interchangeably (as results are almost identical) so only one type of control will be included alongside test compounds.

To ensure accuracy of data, we will also use blinded experimentation for the majority of studies whereby each animal is given a number and the investigating scientist is blinded to which procedures or treatments each animal has received. Unblinding only occurs after the final analysis of tissue and data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will maximise data by analysing as many parameters as possible, using as much tissue as possible. Whilst we may be aiming, for example, to analyse regeneration in the optic nerve, we can also retain complete eye and brain tissue for complete analysis of all the pathways potentially involved / affected.

We will list excess organs, where possible, on the animal facility mailing list. We have shared organs with other groups in the past and will continue to donate extra tissues.

Injury or disease procedures will only be performed on one eye per animal, with the other eye serving as the uninjured control. This also helps to optimise the number of animals per experiment, rather than using additional animals as controls.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

CNS axon injury model: - Mouse optic nerve crush injury.

The optic nerve crush injury is a widely used and widely accepted model of injury to the axons of the central nervous system, as well as being accepted as a model for measuring neuroprotective effects. Effects identified using this model are good indicators of targets or treatments for protecting and repairing both the injured and diseased CNS. Of the available models of CNS injury, it is one of the least severe, but returns many beneficial results.

The optic nerve crush procedure has been used for many years. It causes damage to the axons of the optic nerve which is highly reproducible, and leads to a progressive loss of RGC neurons in the retina, which again is highly reproducible. The optic nerve relays visual information from the retina to the brain. The injury process involves exposing the nerve behind the eye, followed by crushing with a pair of fine forceps for a defined period of time, usually a few seconds. The nerve and surrounding tissues are then replaced, and the injury site heals without assistance.

All of our work identifying potential treatments for repairing or protecting the injured or diseased CNS begin using cell models in a culture dish. The optic nerve crush injury is challenging, but is far less severe or invasive than other brain or spinal cord models of CNS injury. Use of the model allows validation of our treatments, whilst assessing

regenerative and protective effects for the injured or diseased visual system. The optic nerve crush model is a highly beneficial system for identifying, validating and comparing multiple and varied treatments.

A laser intervention to raise intraocular pressure and model glaucoma. An established model in rats.

A laser is used to damage to the trabecular meshwork, the eye's drainage system. This causes a blockage which reduces aqueous outflow from the eye, causing eye pressure to build up transiently. This is reproducible, so has a high degree of success, and has been used extensively by the group. It is only successful in rats, and performs best in non-pigmented animals. The laser settings and parameters required have been standardised and optimised over many years. This will keep complications to a minimum, however if these arise they can normally be treated by maintaining eye lubrication.

The combination of these two models means that we can test potential therapies in both injury and disease scenarios, and in two different species. This is important to demonstrate that treatments can be moved towards developing treatments for humans, and for generating data that can be used for patent applications. For this it is necessary to use accepted pre-clinical models.

Why can’t you use animals that are less sentient?

The objective of this work is to identify treatments for the adult human central nervous system, focusing primarily on repairing the injured or diseased visual system. Adult mammals are required for this work primarily because of the similarity of the visual system to humans, but also because of the comparative regenerative ability of the adult central nervous system (CNS). The regenerative ability of the mammalian CNS declines rapidly with maturity, so that infants have some ability for regrowth after an injury, whilst adults have very little capacity for regeneration. It is critical to use adult animals that exhibit this feeble regenerate ability in adults. Degenerative diseases such as glaucoma also mostly occur in adult and aged patients.

Because we are measuring regeneration, it takes a long time for effects to occur (weeks), so it is not possible for animals to remain under terminal anaesthesia. It is crucial that the injury and disease models have relevance to human disease, because the ultimate aim of the work is to identify strategies that can ultimately be developed to treat human patients.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This is a concise application, with minimal injury and disease models, which will be performed by a limited number of researchers. We will use a recently developed scoring system for recording surgical procedures, on a range of 1-10. Scores below 7 will be followed up with additional care post-surgery, and notes will be made of any particular areas of concern. Animals will be examined throughout the study for evidence of damage

and given post-operative eye-gel, or antibiotic if required. Weight and behaviour of animals of concern will be additionally monitored for a 7 day period. Investigators will be required to maintain these records to improve their techniques, and confirm optimal care of animals.

Researchers train routinely on dead animals to allows them to perfect their surgeries and techniques (dead animals are sourced from excess stock in house, which were destined to be killed). This reduces variability in their studies and helps reduce animal usage through failed procedures and minimises suffering.

For injury or disease models, analgesia is provided during the procedures to limit suffering, and maintained for a day afterwards.

A shared spreadsheet will be maintained within the group which details the course of all animals through all procedures performed. This spreadsheet will record each animal, every procedure performed on that animal, the actual severity reached, additional comments to the study and how the tissues were used and stored. This system has worked very well in the past and we have improved our recording and monitoring of animal welfare.

We will ensure optimal communication between the researchers and the technicians in the animal facility, to educate facility staff and managers about the techniques we are using, so that abnormal or adverse effects can be rapidly identified and treated.

Our wider research group has extensive previous experience with the techniques and procedures in this licence application, and has a comprehensive list of potential adverse reactions that could potentially arise from either these or other procedures involving the eye. This will be made available to all the investigators training for or using the procedures, as well as technicians and researchers who are looking after the animals, in order to aid in identification of adverse effects and to minimise potential harms.

Animals will be housed in groups to enable social interaction and grooming. Animals used in these procedures do not normally fight after surgery and open wounds are not expected. We have experienced minimal signs of stress or discomfort from our procedures in the past and expect a good quality of life for the animals on this licence.

We are also developing new tools which allow for a reduction in the number of eye injections.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow ARRIVE and PREPARE guidelines. Current publications on the LASA website will also be read. There are also best practice guidelines issued by ARVO, the Association for Research in Vision in Ophthalmology. They have issued a “Statement for the Use of Animals in Ophthalmic and Vision Research” which is available on their website. New researchers will be directed to these resources to encourage current best

practice. Further resources regarding veterinary ophthalmology are available through BRAVO, the British Association of Veterinary Ophthalmology although these relate mostly to up to date approaches for treating animal eye disorders.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We have a strong record in cell based models of CNS injury, and will continue to develop these to be as relevant to human injury or disease as possible. In routinely keeping up to date with neuronal cell biology literature we will also be alert to new developments in cell-based neuronal models, with developments arising from studies using stem cell derived neurons, human "mini-brain" organoid type cultures, and patient-derived neuronal cell models of disease. We will also continue to monitor the

NC3Rs website for developments, as well as the Norwegian 3Rs website "Norecopa"and will incorporate new approaches into our research. Up to date surgical advice is available on the LASA website, as well as best practice guidelines from ARVO, the Association for Research in Vision in Ophthalmology, available on their website.

We are also collaborating with labs worldwide who use human and animal models of retinal disease, and will keep up to date with developments in their labs, and incorporate these in our collaborative projects.

158. Analysis of skin carcinogenesis in transgenic mice

Project duration

2 years 0 months

Project purpose

- Basic research

Key words

cancer, transgenic, skin, oncogenes, tumour supressor genes [TSG]

Animal types	Life stages
Mice	adult, neonate, juvenile, embryo, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project uses genetic engineering technology to create transgenic mice that develop benign or malignant skin cancer, and is geared to finish on-going work delayed due to Covid, which are now in the latter stages. Exploiting the latest in techniques, tumours are only induced after topical treatment of skin with steroids to activate the genetic mutation[s]. Thus, any disease is highly localised to specific skin sites and the model can assess effects of several mutations in pre-cancerous stages that lead to malignant tumours in efforts to identify the real mutations and validate the mechanism of how they drive cells through each stage of cancer.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The incidence of skin cancer is rising steadily, and the most significant stage in clinical terms for the patient is the conversion of non-malignant, benign tumours [called papillomas] to malignant ones called carcinomas and their risk of spreading called metastasis. This research study will involve studying the activation of cancer-causing genes [called oncogenes] or inactivation of cancer preventing genes [called [tumour suppressor] genes (TSGs)] in the skin of genetically modified]mice [called transgenic mice]. Using this real life transgenic mouse approach can therefore test which are the key genes thought to be responsible for causing tumours [the “driver” mutations] and by studying their co- operation, we can assess the stage at which mutations in these genes become active to cause cancer. The genes chosen to study represent common genes that are known to help regulate skin cell growth and therefore are likely to go wrong in cancer; and no longer provide the protective systems that naturally have evolved to cope with every day mutations occurring daily sun exposure. Studies of these transgenic mice will aid in identifying the relevant molecules involved in causing cancer and thus become a target for the studies aimed at stopping skin cancers from forming, which may be equally applicable to other cancer types such as breast, colon and lung cancer.

What outputs do you think you will see at the end of this project?

This study is designed to develop genetically modified animals that directly test which genes can drive cancer development in a real-life situation and thus identify targets that can be modified to slow or stop the cancer process and now are mounted by the mouse skin to help prevent benign tumours from forming or stop their progression to become malignant cancers- which is the most important stage.

This mouse model of cancer formation has a particular strength in its ability to investigate the events that underlie both the early stages to form benign tumours and what causes their progression to become malignant. Hence, a study of how the mutations cause progression will both identify the suspected driver genes and test if key tumour suppressor genes [TSGs] can stop the process or slow it down. As a bonus, this type of study will advance the understanding of how these genes work in normal processes such as normal skin growth and how the epidermis (the upper part of the skin) helps maintain the integrity of the skin.

All methods required to regulate expression of genes in the epidermis are well established and have been successfully exploited in the past to identify and test the driver mutations and explore how they interact to create cancers.

Who or what will benefit from these outputs, and how?

This mouse skin model of multistage cancer formation is considered one of the classic ways to study cancer and it is envisaged that these results will be relevant to other cancer types; e.g. breast, lung and colon. All the results are maintained on database available to other researchers; and all biopsy specimens are stored as frozen samples or fixed tissues ready for new experiments.

The mice are available to other scientists investigating cancer and anti-cancer therapies. All methods required to establish genetically modified transgenic mice are well established and have been successfully exploited to validate mutations and explore their effects.

Ideally, in the long term, identification of the pathways involved in cancer formation could lead to the discovery of new strategies for skin cancer, one of the most common cancers in the world, which may be equally applicable to non-skin cancers.

Further, the animals themselves would provide a good way to stringently test new treatments that target these known mutations, in a real-world situation, prior to clinical trials in humans.

How will you look to maximise the outputs of this work?

Findings will be presented to other scientists through publication in peer-reviewed journals and presentations at scientific conferences and meetings. Under the previous project, six papers have been published and peer reviewed data has been presented at many international scientific meetings.

The Institute has a data repository that includes full text of my published journal articles and all conference proceedings. In line with RCUK requirements these data remain accessible for 25 years.

The mice and tumour biopsies are also shared between collaborators in the UK, Europe and the USA.

Species and numbers of animals expected to be used

- Mice: Breeders 1,200;
- experimental 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The mouse skin model of multistage cancer formation is considered one of the classic models for cancer research. Development of skin cancer in mice has a particular strength in its ability to investigate each stage of tumour progression, from early pre-cancers [termed skin hyperplasia] to benign tumours [termed papillomas and keratoacanthomas] that may regress or progress into fully malignant tumours (termed squamous cell carcinomas [SCC]).

Being located on the skin, due to the ability to target expression of mutant genes specifically to the skin, these tumours are easily observed and any changes are easily followed over time.

This has allowed scientists to analyse how tumours progress in adult animals; and test which mutations can drive each stage of cancer formation- or not and identify which tumour suppressor genes [TSGs] fail.

In particular, this allows a focus of this project to assess what causes a benign tumour to become malignant- which is the most important stage for any cancer patient.

Typically, what will be done to an animal used in your project?

Individual transgenic mouse genotypes have been established on the previous project licence and transferred to this project licence. These are routinely bred to create new genotypes. In these mice, one of the genes is a gene switch regulator which is turned on when skin is painted with a steroid. In adult mice which have been bred to contain both the gene switch regulator and a target gene, expression of the target gene only occurs in areas of skin treated with a steroid.

This genetic engineering can therefore activate a mutant cancer causing [oncogene] gene or inactivate a target TSG expression or activate new TSG expression in attempts to inhibit cancer growth.

Thus, potential tumour formation can be highly localised by painting an ear tip or lower back and may develop tumours at these sites; or tumour growth may be inhibited depending upon which genes are turned on.

Typically, juvenile mice [@ 24-28 days] are tagged at the ear and a tiny piece of tail tip is removed for DNA analysis to identify which mice contain the correct genes. These breeders are maintained for up to 6 months.

Once tagged mice are identified with the correct genes, they are anesthetised and treated topically with the steroid at the ear tip and also on the lower back; which has been shaven.

These mice are then maintained for up to 6 months to assess tumour inhibition or benign tumour progression.

What are the expected impacts and/or adverse effects for the animals during your project?

Adult animals that have been topically treated with the steroid on each ear tip and lower back are most likely to develop tumours at these sites; as seen in earlier studies. Initially, this manifests as a scaly, thickened skin and then may form a small, wart-like tumour by 8-9 weeks of age. This may or may not convert to become a malignant tumour over subsequent 4-6 weeks.

These inducible mutations are applied to mice that express a cancer initiating gene or a cancer promoting gene exclisively in the epidermis-the outermost layer of the skin - but not in any internal tissues. In addition, these foundation mice do not express these genes in the tail but can over a period of months develop benign tumours at the ear tag site as benign tumours are induce by the tiny ear tag wound. These small benign tumours do not progress to become malignant and thus provide the ideal foundation to assess which new gene[s] will cause malignancy or ideally identify the genes that can inhibit this event- as for any patient, the conversion of benign tumours to malignant is the most significant event in their disease.

Thus the goal of this project is to assess which genes drive formation of these tumours and another goal is to add or remove genes that inhibit this process- and thus some inhibited mice may be maintained for up to 6 months to assess if this approach was successful.

By employing the ability to induce mutations at specific times via topical steroid treatment, the majority of breeders [>80%] established in the previous PPL did not display a harmful phenotype; with the remaining 20% possibly exhibiting a benign ear tag tumour by 3 -6 months of age.

Tail tipping is carried out under general anesthesia using aseptic technique to provide a tissue sample for DNA analysis, as sampling from this location does not induce tumours whereas sampling from other anatomical locations can induce the formation of tumours in the strains of transgenic mice employed.

Any mouse developing an unexpected harmful phenotype will be humanely killed by Schedule 1.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice:

- Mild (80%),
- Moderate (20%)

What will happen to animals at the end of this project?

Killed

Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The objectives of this project cannot be achieved without the use of live, genetically altered [GA] animals. It is the goal of the project to establish mouse skin models to verify causal roles for relevant mutations that drive tumour progression and identify sentinel systems that have evolved to inhibit cancer.

Which non-animal alternatives did you consider for use in this project?

Experiments involving tissue culture using mouse or human skin cells and transformed by introduction of genes [transfection] have been performed. Transformation is assessed by growth from low numbers, ability to migrate across the dish, seed new colonies and resistance to cell-death.

Why were they not suitable?

Unlike cell culture systems, mice (including those genetically altered) offer the possibility to determine the influence of factors critical to cancer progression such as a blood supply, the immune system, and the growth controls of cells that together with the physical barriers of tissues help to prevent the invasion by tumour cells. However, experiments will utilise skin cells from transgenic mice for tissue culture experiments and the results compared to the mouse data to assess whether cultured cells accurately mimic progression of tumours observed in animal models sufficiently well to replace the need for monitoring malignant progression in adults.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Considerable experience has been gained in previous experiments over the past 25 years that have distilled the numbers required up to 15 animals per genotype in repeat experiments for up to 6/7 months. Given the requirement for topical treatment to activate the mutations, e.g treating each ear tip and a small dorsal area, this has the capacity to produce up to 90 biopsies from these 30 mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Whilst generating these cohorts requires breeding of multiple GM lines, breeding strategies are carefully structured and rigorously monitored to ensure that only the

minimum numbers required for statistical power are generated. In addition, given the requirement for topical steroid treatments, animals carrying multiple target transgenes can be routinely maintained without phenotypes; these act as a foundation source reducing overall numbers required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Untreated breeders act as additional controls and since several genotypes have already been characterised repeating these comparison controls is unnecessary; thus further reducing numbers required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse skin is considered one of the classic models to study cancer formation. This has been combined with production of transgenic mice to assess effects of mutations in genetically modified animals produced by standard techniques. A major strength of this approach employs a skin-specific, inducible gene switch system that prevents disease during animal development or unnecessary disease in internal organs. Thus, during breeding and juvenile development, tumours do not appear; and moreover, as tumours can be highly localised [e.g. treatment of ear tip] disease is further minimised.

Another major advantage of skin models is their accessibility, which not only facilitates induction of localised disease via topical treatments, but also allows macroscopic observation of tumour formation or tumour progression without invasive procedures.

Why can’t you use animals that are less sentient?

The objectives of this project cannot be achieved without the use of adult, genetically altered [GA] animals. It is the goal of the project to establish inducible transgenic mouse skin models to verify causal roles for multiple, relevant mutations that drive stage-specific tumour progression, explore the subsequent mechanism and identify putative compensatory sentinel systems that have evolved to inhibit carcinogenesis.

Mice are the mainstay of this kind of approach with many relevant models published with which to compare data and acquire appropriate reagents from colleagues.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

This project employs a skin-specific inducible gene switch system that avoids disease during animal development or unnecessary disease in internal organs. Thus, during breeding and juvenile development, tumours do not appear; and moreover, as disease can be highly localised [e.g. treatment of ear tip] suffering is minimised.

The accessible nature of skin models allows careful monitoring of all potential tumours without invasive procedures and permit experiments to be terminated as soon as significant data has been obtained.

Animals are regularly monitored and for individuals with possible malignant tumours animals are monitored daily and adverse effects closely monitored to ensure no tumour exceeds the recommendations of Workman et al, 2010 and that we implement pre-set humane end-points.

In addition, to help control pain animals about to undergo the genotyping protocol via ear tagging and a tail tip biopsy are placed on analgesia in the drinking water for 24hous pre and post the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Workman et al 2010. BJC www.NC3Rs.org.uk

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

www.NC3Rs.org.uk; consultation with Named Animal Care Welfare Officers NACWO

159. Mechanisms regulating neural stem cell activity and neuronal production

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Stem cells, Neurogenesis, Quiescence, Ageing

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To identify mechanisms that control the activity of neural stem cells and the process of neurogenesis in the embryonic, adult and ageing brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Neural stem cells play a central role in the generation of neurons and glial cells during brain development and in the adult brain. Defects in neural stem cell activity in the embryo has been linked to neurodevelopmental disorders and defects in adults and in particular in ageing and diseased individuals, to cognitive impairments and mood disorders. Identifying the mechanisms that control the activity of neural stem cells and the process of

neurogenesis is essential to elucidate the aetiology of these disorders and understand how the brain is formed and maintained.

What outputs do you think you will see at the end of this project?

The project will result in an improvement of our understanding of the mechanisms that control the activity of neural stem cells and the process of neurogenesis, and how these mechanisms change between embryonic and postnatal development, adulthood and ageing. The outputs will take the form of publications in peer-reviewed journals and presentations at academic conferences but will also include dissemination to the lay public via popular science initiatives. Materials, data and methods will also, where appropriate, be accessible online.

Who or what will benefit from these outputs, and how?

The main beneficiaries of our research in the short and medium term will be other scientists working in the fields of brain development, brain plasticity and brain ageing. For example, scientists may investigate whether the mechanisms we uncover are deficient in neurodevelopmental pathologies (e.g. lissencephaly), neurodegenerative pathologies (e.g. Akzheimer's disease) and mood disorders (e.g. depression). In the longer term, these studies may lead to the identification of new targets for the development of new pharmaceutical and therapeutic interventions in these pathologies.

How will you look to maximise the outputs of this work?

We will share our data, analysis tools and resources with other scientists, using one to one interactions and collaborations, repositories and our lab webpage, which will reduce the number of replicated experiments in the field. We will disseminate our research by publishing results in peer reviewed journals. We will also present our work to academic peers at scientific conferences (national and international) and engage with public partners to disseminate our results.

Species and numbers of animals expected to be used

- Mice: 20,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will perform our study in mice because of the number of available mouse lines that are genetically suitable, the ease of generating new mutations or manipulating gene expression acutely in this species, and the comparison with previous studies in the scientific literature. Also the brain of mice is sufficiently similar to that of humans to make

its study worthwhile to learn about mechanisms of brain development and function in humans. We will use embryonic, postnatal, adult and aged mice, since we are investigating the changes that occur in neural stem cell activity and neurogenesis during the lifetime.

Typically, what will be done to an animal used in your project?

Some animals will receive injections of substances to induce gene deletions or measure cell proliferation. Some animals will receive several injections with the same or several substances, at variable intervals. They will then be humanely killed at various times after these manipulations in order to study their brain tissues.

Some animals will undergo surgery where a window is opened in their skull for injection of substances into the brain to manipulate gene expression locally. Adverse effects after the surgery may reach moderate levels of severity for a short period of time, but all animals will receive pain relief and will be closely monitored until they recover completely. At variable time after this, they will be humanely killed in order to study their brain tissues.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals will undergo surgery of moderate severity. After recovering from general anaesthesia, mice will be less active for a day or two after surgery. Animals might lose some weight but will typically regain that weight within two to three days. Some animals will also be allowed to age (up to 24 months) and as a result may experience certain discomforts associated with the ageing process. At the end of experiments, or if mice show signs of ill health, distress or suffering that are not improved or resolved within a timeframe approved by the veterinary surgeon, they will be humanely killed.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- 60% of mice will experience a severity category 'Mild'
- 40% of mice will experience a severity category 'Moderate'

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Mice will be used in this project. Neural stem cells can be maintained in culture and we perform such experiments, but their behaviour in vitro is substantially different from what it is normally, as the cells in vivo are embedded in a tissue comprising multiple cell types that interact with stem cells and influence their behaviour. This stem cell niche is too complex and not sufficiently characterised to be reconstituted in culture, and in vivo experimentation in whole animals is therefore unavoidable to reach a thorough understanding of how neural stem cell behaviour is regulated.

How ageing affects neural stem cells and neurogenesis can only be studied in vivo as there is strong evidence that the ageing process, both healthy and pathological, involves interactions between brain cells and the immune and vascular systems, which again have not been reconstituted ex vivo.

The proposed studies could not be undertaken in non-mammalian species because their neural stem cells and niche environment are too different from neural stem cells in mammals and particularly humans (for example, non-mammalian species do not have restricted stem cell niches in the parts of the brain called dentate gyrus and subventricular zone as both humans and mice do).

Which non-animal alternatives did you consider for use in this project?

This project will be done in parallel with studies using non-animal alternatives, including Mouse Neural Stem Cell (NSC) cultures.
Human Induced pluripotent stem cells (iPSC)-derived neural cultures, including brain organoids
Human foetal brain material from the Human Developmental Biology Resource.

Why were they not suitable?

The cell culture models will provide us with useful information. Mouse NSC cultures will be used to get insights into the intracellular mechanisms (e.g. transcription factors, chromatin remodelers) that regulate neural stem cell behaviour. NSC cultures are a reductionist model that allow access to fundamental properties and constituents of neural stem cells that are maintained in this artificial setting (e.g. transcriptional and epigenetic mechanisms controlling NSC activity and quiescence). Other NSC properties differ between culture and in vivo settings, including their interactions with other niche components (absent in culture), their behaviour (e.g. NSC divide asymmetrically in vivo but symmetrically in vitro) or population dynamics (NSC numbers and activity rates change with age in vivo while NSC cultures cannot be maintained long enough to assess these changes), and must therefore be studied in vivo. Human Induced pluripotent stem cells (iPSC)-derived neural

cultures will be used to determine how much similarity or divergence exist between human and mouse neural stem cell and the mechanisms that regulate their activity. However any in vitro result will need to be validated by studies of neural stem cells in mice in vivo because the cells are substantially different in the two environments.

Moreover, in vitro experiments do not allow the study of interactions between neural stem cells and their niche environment, crucial for the regulation of neural stem cell activity, and interactions with the immune and vascular systems, crucial in brain ageing.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers of animals have been estimated based on previous experience when dealing with complex genetic crosses in mouse breeding as well as estimates on the number of experimental animals required based on expected effect sizes and variablity (SD) from the literature, past work, and in vitro pilot experiments.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Several factors will lead to a reduction of animal numbers, including reducing variation and good experimental design involving the use of appropriate statistics. In particular, statistical tests will be used to ensure that we use the minimum number of animals possible to reliably interpret our data.

We will design our mouse breeding strategies carefully to minimize the number of generations necessary to reach the desired combination of transgenes/ mutations, and to maximise the proportion of offspring carrying this combination of transgenes in the litter. Where appropriate, we will use otherwise unwanted offspring as negative controls.

We will also interrogate gene function by performing as much as possible acute manipulations of gene expression, e.g. by injecting viruses or CAS9/gRNAS to avoid the extensive breeding required to generate and maintain mouse mutant lines.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding and holding lines as frozen down embryos and sperm will be used to minimise the number of mice being produced for these studies. However, generation of

surplus GA mice is sometimes difficult to avoid, e.g. when the genotype of interest is homozygote and breeding generates heterozygotes in addition to homozygotes, in numbers too large to be all used as breeders. Where possible, genetically altered lines will be maintained in a homozygous state, thereby obviating the generation of a large excess of offspring with inappropriate genotypes. In other cases, homozygotes will be generated from heterozygote intercrosses, with littermates genotyped as heterozygous or wild type used as age-matched controls. Genetically modified lines will be sourced from repositories to avoid remaking of lines whenever possible. Pilot studies will be undertaken to ensure the correct time course and experimental paradigms for our experimental purposes before larger scale studies are undertaken. Tissues sampled from the animals used in this project will be shared among lab members and with researchers in other labs when applicable.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice have been chosen for this work as the least sentient species to model the human nervous system well enough for our work to be relevant to understanding of human brain development and adult neurogenesis. Mice are essential because the experiments require the use of the latest and most refined gene manipulation technologies to identify and label cell subtypes and allow cell specific gene overexpression and downregulation approaches, and these approaches are available in this species.

Our transgenic mouse models are not expected to exhibit any harmful phenotype. Acute manipulation of gene expression in the brain by stereotaxic injection of various substances (viruses, shRNA, gRNA) will require opening of a cranial window under general anaesthesia. For this surgical step, appropriate anaesthetic/analgesic regimens and post-operative care will be used to minimise pain and surgery will be performed following aseptic technique to reduce the risk of infections. The initial surgery is the only step in which any pain may occur. Any surgical procedures will undergo regular review to identify further refinements to minimise animal suffering including optimisation of implants and anaesthesia/analgesia regimens.

Some of the mice will be analysed by perfusion of the brain tissue under terminal anaesthesia followed by histological analysis. Other mice will be analysed by harvesting the fresh brain tissue after schedule 1 killing, followed by biochemical analysis. The analysis will therefore generate minimal suffering.

Why can't you use animals that are less sentient?

Mice will be used for this project as they represent the least sentient species appropriate for this type of work. Other species which are less sentient (such as fish or amphibians) cannot be used because they do not show sufficient similarities to humans (e.g. they have large adult neural stem cell populations that are significantly different from those found in mammals as their brains continue to grow throughout life).

Some experiments will be conducted in animals at embryonic or early postnatal stages, but other experiments will require using adult and ageing animals because part of the project focuses on stem cell activity and neurogenesis during adulthood and in old age.

Some of our experiments will be done using tissues from animals that have been euthanised or terminally anaesthesised. However, in order to examine the impact of gene expression manipulations, other experiments will be performed on anaesthesised animals followed by recovery for various duration until the time of analysis.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The animal facility staff run a comprehensive health-monitoring programme whereby animals health and welfare are checked and recorded on a daily basis. The animals will be maintained under conditions where their health status can be protected as far as is reasonably practicable. We use refined holding techniques for the animals, as well as group housing and enrichment. We use appropriate anaesthetic/analgesic regimens (including pre- and post-operative analgesia) and aseptic technique to minimise pain and will refine these with advice from the Named Veterinary Surgeon to ensure that we are using the best possible option given our experiments.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Resources hosted on the NC3Rs website, in particular:
- ARRIVE guidelines on experimental design and reporting results.
- PREPARE guidelines for planning animal experiments
- 'Procedures with Care': 'Aseptic Technique in Rodent Surgery'.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs from several sources:
- Liaison with our animal care staff and NC3Rs representative
- Technological advances in the published scientific literature
- The NC3Rs website

160. Information processing in neural circuits

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

neural circuits, sensory processing, mental illness, novel therapies, optical methods

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to find out how activity in identified neural networks enables internal representation, processing, and storage of sensory information in the brain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Improving our understanding of how the brain processes information coming from our senses is in the first instance a matter of considerable, fundamental scientific interest. In the longer term, the insights gained from this project should allow us to identify new

therapeutic targets for mental illness that affects sensory processing (e.g. schizophrenia or ADHD) and storage of sensory input into memory (e.g.

Alzheimer's disease and dementia).

What outputs do you think you will see at the end of this project?

The project will generate output in the form of peer-reviewed research findings; non-specialist public communications; leads for novel treatment strategies for mental illness.

Who or what will benefit from these outputs, and how?

The main beneficiaries will be other researchers studying how the brain processes information from our senses in health and disease. Through the development of potential new treatment strategies, researchers in industry and ultimately the general public stand to benefit from the outputs of this project.

How will you look to maximise the outputs of this work?

We have been very successful in the past to maximise outputs and their impact through varied multi- and inter-disciplinary collaborations. These collaborations typically produce benefits and outputs bigger than the sum of their individual contributions.

Species and numbers of animals expected to be used

- Mice: 8500 (some animals may be purchased-in instead of bred under authority of the license)

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The brains of mice process information from their senses, such as touch, taste or smell, very much like in humans. The use of mouse models for behavioural testing has been refined over many decades.

Uniquely, mouse models combine these properties with the possibility to use very specific, targeted way using genetic modifications. These modifications allow, for example, to manipulate brain activity in a very precise manner or model human neuropsychiatric disease through the transfer of human disease risk genes to the mice. To map connections between brain areas, we will require the use of early life stages. All behavioural experiments will be carried out in adult animals.

Typically, what will be done to an animal used in your project?

Part of the project will involve the generation of genetically altered mice to allow us to investigate the functions of particular molecules and cell-types in sensory processing. These animals are expected to be not fundamentally different in the way they behave from wild-type controls and thus expected levels of severity will be mild. In about 5% of the animals, we will need to trim some of the whiskers of the animal to be able to relate neural signals to touch sensation in specific whiskers. This has minimal effect on animal well-being. In about 30% of the animals it will be necessary to inject substances into the brain to deduce anatomical structures and function or implant electrodes or viewing windows. This will be carried out under general anaesthesia typically lasting no longer than 90 minutes, in aseptic conditions, with about 5% of the animals being humanely killed before regaining consciousness (e.g., where tracer substances require very short incubation time) and the other animals being recovered with appropriate post-operative care and only causing moderate amounts of discomfort to the animals in the study. The behavioural tasks we will use to record conscious, sensory perceptions are painless. In some cases, it will be necessary to motivate the animals to perform these tasks by rationing their food or water during testing. This may result in a temporary weight loss, but this will always be monitored carefully, and extra food or water provided if this occurs. Rationing results in lean, motivated animals and will typically last about 14 weeks. The availability of modern techniques for monitoring or altering neural activity in particular regions of the brain make it possible to carry out all of this work in a manner that should cause only moderate amounts of discomfort to the animals in the study. For example, surgical operations for implantation of ultrafine microelectrodes or for inserting genes into the brain will be carried out under general anaesthesia, in aseptic conditions, and with appropriate post-operative care. The adverse effects that may occur following surgery include transient pain and bleeding, but their incidence is likely to be less than 5% of the animals undergoing surgery. Chronic implants for recording neural activity or for delivering flashes of light for the purpose of altering that activity are small and light- weight, and do not materially affect the animal's quality of life. A relatively small percentage of animals (about 12%) will be used in tests where the head needs to be fixed to enable stable recordings of brain activity. These recordings typically last about 45 minutes per day and are repeated daily for 2-3 weeks. In these tests, the animal is supported on a moveable platform that allows the animal to perform behavioural tasks, for example, navigating through a virtual maze projected onto screens. This method is now very established and well tolerated by mice displaying the same behaviour as when walking freely and should cause only mild amounts of discomfort to the animals in the study. Animals will be killed humanely at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals will experience transient, post-operative pain, typically lasting no longer than 1-3 days during which pain relief will be provided. Some animals will be fitted with chronic head implants (typically just a small, light-weight and thin, implanted window in the skull) for typically about three months or until the scientific end is reached. In our experience, these implants do not noticeably affect animal welfare or behaviour.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals (>50%) will be used for breeding and experience sub-threshold severities. Of those animals used in procedures beyond breeding, about 5-10% will experience Mild severity and about 30-35% Moderate severity.

What will happen to animals at the end of this project?

Killed
Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our project investigates how the brain processes information from the senses. Currently, this can only be studied by using the brains of animals or humans, as our understanding of brain function is too rudimentary to generate realistic mathematical models for testing. Brain imaging measures in humans lack the sensitivity to observe changes in the properties of individual, identified brain cells in response to sensory stimuli. Moreover, a key aim of this project is to try manipulating brain activity in a very controlled, targeted fashion using genetic modifications to specific brain cells, which is not available in humans. Additionally, we aim to relate brain cell activity to the underlying neural circuitry at a microscopic level. This requires the use of post-mortem histological measurements, which would not be ethical or practical to carry out in humans.

Which non-animal alternatives did you consider for use in this project?

We considered lower animals (e.g., c. elegans), human cell in vitro models and mathematical models of brain function.

Why were they not suitable?

Lower animals or human brain cell in vitro models do not nearly model human brain function in health and disease sufficiently as well as the mouse. Too little is known about brain function in general, to make mathematical models a suitable replacement given the project aims.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Yes. Calculations have been carried out to determine the necessary number of animals for each experiment, ensuring significance of our results but also minimising the number of animals used. We are additionally able to keep animal numbers to a minimum by using cutting edge methods, such as optical recordings of many hundreds of identified brain cells at a time, that yield large amounts of data and experimental designs that allow multiple measurements to be made from each animal.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We estimate effect sizes and adjust required numbers of animals to the minimum required to test our working hypotheses. Furthermore, we employ within-subject experimental designs wherever possible to reduce the number of animals required.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will work closely with our animal facility to ensure efficient breeding strategies. Additionally, we will use pilot studies when using new approaches to estimate effect sizes. We will also continue to make use of computer modelling of brain activity to reduce the conditions we need to be explore in experiments, thereby reducing the number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice will be used because they are the animals with a sensory system that is comparable to that in humans.

The impact on animal welfare will be minimised by carrying out procedures in state-of-the-art facilities and using best practice methods. Breeding and colony maintenance, including genetically altered mice, will follow the Home Office assessment framework for efficient breeding and maintenance. We will only use genetically altered mice that exhibit a mild phenotype (e.g., with no effects on feeding or welfare) or no measurable behavioural phenotype (e.g., mice producing a fluorescent marker in certain brain cells). Where animals undergo surgical operations, these will be carried out very carefully under anaesthesia and aseptic conditions, and the animals are given painkillers and will be closely monitored until they have fully recovered.

Sometimes it will be necessary to regulate the food or water intake in mice in order to motivate them to perform behavioural tasks for a food or water reward. We have very strict guidelines in place to mitigate any harm from this food or water regulation, as well as for the behavioural tasks used.

The use of state-of-the-art methods will help reduce the impact on animal welfare while, at the same time, increasing the amount of scientific insight that can be obtained from each experiment. These methods include, for example, the introduction of genetic modifications to specific neurons, allowing us to change their activity in a precise fashion.

Animal welfare is regularly monitored. Clear instructions for all protocols ensure that procedures are aborted before exceeding set limits for impact on animal welfare.

The data obtained from these experiments will be used to refine computer models of the brain that will help to guide subsequent experiments and contribute to a reduction in the number of animals needed.

Why can’t you use animals that are less sentient?

Our project aims to unravel the biological principles of sentience, so the basis for feeling and perceiving things using our sense, itself. Therefore, a minimum level of sentience is required in the animals used in the project. Furthermore, we hope to extrapolate our findings in mice to the brain function of humans, particularly in neuropsychiatric conditions, and therefore require animals with a minimally similar brain architecture.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We employ increased monitoring whenever there are possible welfare costs to the animals. For example, animals receive daily post-operative care, including pain relief where necessary, for at least one week after surgery. We also have strict, staged SOPs for habituating animals to handling staff and experimental apparatus, thereby minimising potentially stressful situations for the animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We regularly review our procedures and SOPs against the published literature, particularly peer- reviewed protocols (e.g., Nature Protocols) and use the published resources on the NC3Rs website.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We keep abreast about advances in the 3Rs through regular newsletters from our Home Office Liaison, NVS, and NACWO. Group members involved in the project also attend relevant workshops and online courses. Furthermore, 3Rs and license matters are reviewed and protocolled in a separate agenda item in our weekly labmeetings.

161. The regulation of platelet function as a central mechanism for the control of cardiovascular function and development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Platelets, Thrombosis, Heart attack, Stroke, Obesity

Animal types	**Life stages**
Mice	adult, embryo, juvenile, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand the roles of specific proteins (e.g receptors, enzymes such as kinases, phosphatases, cyclases, ion channels and other intracellular cell signalling proteins) in (A) the regulation of the function of platelets, and the implications of these in blood clotting and thrombosis, and

(B) their roles in additional processes in which platelets are implicated including obesity, vascular and lymphatic development, vascular remodelling, and inflammation. Using our increased understanding of the processes that control platelet function in health and

disease, we aim to establish more effective strategies and medicines to prevent thrombosis and other conditions in which platelet function is implicated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

When blood clotting occurs within blood vessels, a condition known as thrombosis that is often triggered as a result of blood vessel disease, this prevents the flow of blood to tissues and organs that lie downstream. Thrombosis is the principal cause of heart attacks and strokes. Platelets are blood cells that initiate blood clotting, whether to prevent bleeding or cause thrombosis. While considerable success has been achieved in recent decades in the development of drugs that target platelets to prevent thrombosis, currently available treatments are ineffective in many patients, while others suffer serious side effects including bleeding. Understanding of the complex mechanisms that regulate platelets in health and disease is important for the development of more effective, more refined, and more specific and safer anti-platelet medicines to prevent or treat thrombosis. The ability to adjust platelet reactivity in an individual, potentially through targeting multiple processes, will enable more precise control increasing efficacy thereby reducing the likelihood of thrombosis, while maintaining effective blood clotting where this is needed. This project is expected to result in the identification of new drug targets, understanding of the effects of genetics and chronic conditions such as obesity on pre-disposition to thrombosis, and characterisation of combinations of mechanisms that may be modulated by new medications.

This work complements extensive analysis of the regulation of blood clotting in humans by the applicant, and the development of new analytical and mathematical modelling methods to provide treatments targeted to specific patients (precision medicine).

What outputs do you think you will see at the end of this project?

At the end of this project we will expect to have

1. Identified new mechanisms, and the proteins in platelets that control these, which control the functions of platelets, and therefore regulate blood clotting.

2. Established which of the mechanisms identified in (1) are essential for proper blood clotting, and which may be involved in abnormal blood clotting which leads to heart attacks and strokes.

3. Determined which of these mechanisms or proteins may be effectively targeted in the development of new medicines to prevent heart attacks and strokes.

4. Discovered the effects of obesity and associated metabolic disease during pregnancy on platelet function and therefore levels of blood clotting in offspring when also exposed to a high calorie diet. This will provide mechanisms to help design new therapies to combat the impact of poor diet in pregnancy on future generations.

Who or what will benefit from these outputs, and how?

The key benefits will be to human health. Cardiovascular diseases (CVDs) are the single largest category of disease which leads to poor health and death in the UK. In the last year around 166,000 deaths were caused by CVDs, with a large proportion due to abnormal or un-necessary blood clotting. This equates to a heart attack every 8 minutes in the UK. The knowledge gained from this work will lead to the development of new medicines or measures to reduce the risk of developing CVD, and better therapies to treat those already affected. This work will be associated with extensive population and clinical studies, which allow our discoveries in mice to be transferred to human benefit. Following the identification of new target proteins or processes within platelets that may form the basis of new anti-thrombotic strategies, we would anticipate the development and preclinical testing of new drug candidates to take between 5 to 10 years. Platelet regulation is complex, and some experiments may lead us to conclude that a mechanism should not be targeted to prevent disease, and knowledge of this will be equally important for researchers of blood clotting to understand.

Abnormal platelet function can cause serious disease symptoms that are less obviously related to the mechanisms that cause heart attacks and strokes but are nonetheless life-threatening. Our work has identified why and how blood clots form in the lungs and other organs of patients with severe COVID-19 infection. We have indentified abnormalities in the immune responses of these patients to the COVID- 19 virus that trigger excessive clotting. Importantly we have also identified existing drugs that specifically prevent the abnormal clotting in the laboratory, and are currently testing the effects of these in clinical trials. This example demonstrates how our research may lead to new treatments that we hope will prevent deaths that are caused by abnormal blood clotting.

We will disseminate all of our research findings to other scientists working in this field through research publications and conference presentations, maximising the potential contribution and translation of our work for human benefit. Where appropriate we will also discuss our work with the general public, engagement with which has increased substantially due to the COVID-19 pandemic.

How will you look to maximise the outputs of this work?

A number of avenues will be used to maximise the outputs from this work. This will include publication in high ranking scientific journals, and presentation at internationally leading research conferences.

Within the research group we are experienced in the art of drug discovery through high throughput screening and the development of biologics, and therefore will be able to take

promising aspects of the work forward in the development of new medicines. We interact extensively with industrial researchers in the pharmaceutical and biotechnology sectors, and have well developed networks to explore the development of therapies based on our work. Examples of this include work to explore the potential re- purposing of existing new drugs used in the treatments of haematological cancers for the prevention of thrombosis, refinement of drugs to reduce myeloma treatment-associated thrombosis, and the repurposing of immune-modulating drugs to prevent COVID-19 associated thrombosis (with clinical trials underway).

Due to the important potential impact on human health that is associated with our research, we frequently engage with the media to inform the public of our research and the importance of measures that may reduce risk of CVD. Continued interaction with the media in association with this project is envisaged, particularly where the outcome of work (for example experiments to determine the effects of maternal obesity on blood clotting levels) may lead to new public health advice policies.

Species and numbers of animals expected to be used

- Mice: 5,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To determine the roles of specific platelet proteins and the processes that these control in bleeding and clotting diseases we presently have no alternative than to test this using animals, noting that all research prior to this is conducted using blood obtained from humans. The similar circulatory systems in humans and mice, and very similar physiology of blood platelets between the species has led to the mouse being adopted internationally as the most appropriate animal in which to study platelet function in the complex circulation in health and disease. This also holds the major of advantage of being able to use genetically altered mice, which allows questions to be addressed that would not otherwise be possible. This is because platelets lack a nucleus, which prevents their production in cell culture or the use of molecular biology techniques to manipulate the levels of specific proteins, or introduce mutant forms of a protein. The experimental approaches that we have established using mice have become acknowledged as the principal pre-clinical experimental approach in the development and testing of drugs to combat thrombosis and related disorders. We have led the establishment and refinement of these experimental approaches in the UK, working closely with colleagues internationally who were responsible for the development of some of the laboratory technology that we have implemented, and others who have also adopted these techniques in locations around the world. The wide adoption of this approach

internationally allows us to compare our experimental outcomes and data with the wealth of published literature, and collaborate more effectively with other researchers.

The breeding of mice involves the use of young adult mice, and the generation of juveniles that are either used for further breeding or within our experiments. Bleeding assays may use adult mice at any age, although most of our techniques to analyse thrombosis require young adults to be used.

Typically, what will be done to an animal used in your project?

The most commonly used procedure in this project (up to 5000) will involve the breeding of mice that have been genetically altered, so that either specific cells or all cells in the body either lack a specific protein, gain a specific protein or a protein is replaced with a mutated form of a protein. This allows us to understand the role of this specific protein in health and disease. In most cases pregnancy progresses normally (21 days) and mice grow and suffer no illness, pain or stress. In some cases altered blood clotting may cause adverse effects such as uncontrollable bleeding, so mice are observed very carefully, even though breeding of mice is routine and straightforward. Genotyping is performed to assess the genetic makeup of mice, and for this a small piece of tissue is obtained from the ear, which is considered a minimally invasive way to obtain genetic material. Where ear notching is used for indentification, removed tissue will be retained, resulting in no additional procedures being required for genotyping.

The mice that we breed, and sometimes mice imported from other laboratories are used to determine whether thrombosis is reduced or increased and whether normal blood clotting is compromised.

Bleeding assays (up to 1500 mice over 5 years) are performed by making a small incision at the tip of the tail and measuring blood loss or the time that it takes for bleeding to stop. This takes no more than 30 minutes to perform and mice that continue to bleed after 20 mins are killed humanely. This surgical procedure is performed under general anaesthesia and in most cases mice are not allowed to wake up following this. Rarely, animals may be allowed to recover and used subsequently to measure thrombosis. Thrombosis, another surgical procedure is also measured under general anaesthesia without recovery (up to 1500 mice over 5 years). This may be achieved in a number of ways, following dissection of specific vessels to that they can be observed using a video microscope, and thrombosis is triggered either by using a laser, or by application of chemical to a vessel, or by physically manipulating a vessel. Thrombosis is measured and evaluated by microscopy and sophisticated software for analysis of data. This type of analysis allows multiple measurements from a single mouse, in experiments that last up to 2 hours in duration.

In some cases mice will be treated before blood sampling, or analysis of thrombosis or bleeding, with anti-thrombotic drugs or other agents that are expected to modulate platelet function. These drugs will be injected either into an exposed vein or canulated carotid artery, under the skin or into a body cavity, or administered in drinking water or by oral

gavage. Mice may also be fed a high calorie diet to induce obesity, prior to analysis of platelets.

What are the expected impacts and/or adverse effects for the animals during your project?

In most cases the breeding of genetically modified mice will result in mice that may have mildly defective blood clotting, but are essentially healthy. On occasions it is possible that a genetically modified mouse may suffer from unhealthy bleeding or thrombosis, although in our experience this is very rare. Should this occur and exceed acceptable limits of severity (mild or moderate), mice will be killed humanely as soon as possible.

The experiments that we do with the genetically modified mice to test bleeding or thrombosis will be performed under general anaesthesia, so the mice will not feel anything and in most cases will not be allowed to wake up after the experiment. Some mice used to test bleeding or thrombosis will have been pre-treated with medicines of other agents that are expected to modify platelet function or number, which may result in reduced or increased platelet function and/or blood clotting. This may result in an increased chance of extended bleeding (particularly during procedures) or thrombosis that would cause the mice some pain or distress. Our experience suggests that this would be a rare occurrence, affecting less than 10% of mice treated in this way. Other mice used in these procedures will have been treated to induce obesity. This will result in weight gain or conversely a failure to thrive and consequently a reduction in weight and potentially hair loss and skin irritations. Our previous work indicates that elevated calories eaten by pregnant mothers and their offspring increase the levels of platelet function. Based on previous experiments we do not expect increased levels of platelet function to be associated with any clinical signs in the mice.

On rare occasions (<10% of mice used to measure bleeding) mice will be allowed to recover following analysis of bleeding and subsequently used in experiments to measure thrombosis provided that their health has returned to normal, and that they exhibit no continued effect of the bleeding tests (e.g. re- bleeding) and that they continue to thrive.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The largest scale procedure conducted in this project is the breeding of genetically altered mice (5000 mice), a protocol that has a MILD severity limit.

The measurement of bleeding and thrombosis is conducted under general anaesthesia. All thrombosis testing and most bleeding testing are done without recovery. Less than 10% of mice in which bleeding tests have been performed will be allowed to recover before use in thrombosis tests. The mice used for these protocols may be administered agents or drugs

that modulate platelet function or number. A MODERATE severity category is appropriate, and therefore the severity limit for tests of bleeding and thrombosis is MODERATE although for >90% mice, only MILD severity is experienced.

Some mice will be fed on a high calorie diet during pregnancy. The offspring resulting from these (and females that are not fed a high calorie diet) may also be fed a high calorie diet. This may result in weight gain or loss, and failure to thrive. Adverse effects are expected in no more than 5% of animals and will be used on a protocol with a MODERATE severity classification.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Platelets control blood clotting which is important in preventing uncontrolled bleeding. These cells also trigger unwanted blood clots within the circulation (thrombosis) which causes heart attacks and strokes, as well as a number of additional life-threatening conditions. While we do obtain a great deal of information about platelets and their roles using human blood samples in the laboratory, determination of the role of specific proteins in the control of platelet function and thrombosis development can only be done within an intact circulation. This is because blood within blood vessels is exposed to special conditions such as blood flow and chemical messages from other cells including those of the blood vessels. These tests are particularly important because they will lead to us identifying proteins or processes that may be targeted to prevent heart attacks and strokes. Abnormal bleeding is a potential side effect of targeting proteins that modulate platelet function, and therefore it is also important to established whether bleeding is altered in our mice, to indicate whether specific proteins might make good 'drug targets' which do not cause adverse bleeding.

Sometimes we need to study platelets from mice that have been genetically altered (transgenic) to lack a specific protein, or to produce an abnormal version of a protein. These mice may be used to measure thrombosis or bleeding (as above) or blood samples may be obtained from these mice and studied in the laboratory. Our need to breed transgenic mice stems from the fact that there are no reliable methods of generating platelets in the laboratory from bone marrow cells or cell lines. This means that the only source of genetically altered platelets is from an animal model (work is underway in some laboratories to overcome this limitation and produce platelets for patients, but realising this ambition will require several more years of research). Previous studies have utilised genetically altered mice to identify and delineate the processes involved in platelet

activation. Data obtained from the use of genetically altered mouse models in this application would therefore be directly applicable to published studies.

We have previously shown that platelets in offspring may be programmed by the diet of their mother during pregnancy, making the platelets more likely to cause clot formation, particularly when the offspring are themselves exposed to a high calorie diet. The precise mechanism and duration of this effect is not understood. This has substantial implications for human health, but experimentally modifying the diet during human pregnancy would be neither ethical or practical, and therefore we have established a mouse model to investigate this aspect of our work. The changes produced during pregnancy are complex, and cannot be recapitulated by cells in culture, and therefore we need to induce obesity and metabolic changes during pregnancy in mice. The specific use of mice allows us to take advantage of the similar composition of blood vessels and blood in mice and humans, and to use established models of thrombosis and bleeding (see above) which may be compared with extensive published scientific studies.

Platelets are also involved in a number of important system effects in the body including the development of certain types of vessels (blood and lymphatic) and the control of inflammation in blood vessels and tissues. While much of the underlying biological studies are performed using cells in culture, cell lines and human platelets, the specific processes that regulate these functions require confirmation and detailed study in a whole mammalian system.

Which non-animal alternatives did you consider for use in this project?

Extensive work with human platelets and other blood cells is always performed before we consider using mice to study these cells and their impacts on health and disease.

While presently studies in animals are not completely avoidable we have been working to reduce our reliance on animals in our research, and as a consequence we have been able to make considerable reductions in our planned work with animals in the next 5 years, to approximately 50% reduction in comparison with the project that this replaces. This has accured for a number of reasons that span the 3Rs principles. The over-riding development in our work has been our ability to exploit our current understanding of platelet regulation to develop a more patient-facing and translational focus. This reflects that maturity of the science in this area and a range of technical innovations, including our development of new assay systems, clinically relevant tests and computational and software resources that employ machine learning to analyse complex data. Indeed, these developments have enabled us to respond rapidly to the COVID-19 pandemic resulting the discovery of why seriously ill patients develop blood clots, and the establishment of a clinical trial to test drugs that may alleviate this.

Within the group we have a strong focus on why we do what we do, to assess what is really necessary in order to achieve the aims of our research while ensuring the highest quality of outputs. This is aided by substantial animal research expertise and knowledge of

governance in this area, supported by a team member who has received specialist training in the care and welfare on animals used for research. As scientists we have increasingly pushed back against manuscript reviewers that request work to be repeated but with small changes or the use of a different experimental model or mouse strain. These types of requests are often ill-considered and simply following these views is not appropriate from an ethical perspective. Models are models. They do not necessarily replicate perfectly human disease and biology. So attacking a problem using multiple animal approaches potentially just results in multiple different outcomes and confusion. Therefore, where we consider animal experiments to be necessary, careful planning and selection of appropriate approach is essential, prior to initiation of the work. This may involve pilot studies to establish which approach is apprioriate to test our hypotheses. These principles guide our approach as a group to experiments using animals and decisions are made following considered discussion within the group (e.g. at lab meetings) ensuring accountability and the development of a mindset that challenges and does not follow trends in research without question.

We have also been working for several years on computational approaches which we hope in the future to allow us to further reduce our reliance on animals in our research. We have developed a suite of mathematical models that are able to simulate the activation of platelet function, and the impact of anti-platelet drugs on this. This work, which has been developed with support from the British Heart Foundation is called the Virtual Platelet, and will allow us to conduct experiments “in silico” for the purpose of understanding the basis of variability in platelet function in the population, for drug discovery (including understanding the impact of targeting more than one molecule simultaneously) and diagnosis on the basis of platelet dysfunctions. These models currently include simulations of both human and mouse platelet function, which further allows changes in platelet function in humans measured in vitro to be related to potential impact on thrombosis risk or size. Data collected within this project will contribute to the development and testing of these mathematical models. Importantly these models will also allow us to test hypotheses for which lab-based experiments would not be possible, feasible or ethical using animals. This will in the future allow us to reduce our reliance on the use of animals in our work - a goal which, along with improving the translation of our research to benefit patients, motivates this work.

We are also involved in the development of 'vessel on a chip' experimental approaches to study platelet function ex vivo supported by research funding from several organisations.

Why were they not suitable?

Whole systems approaches are the only way currently to test the importance of specific proteins and their mechanisms of regulation of platelet function, with respect to diseases in which platelets are involved because of the complexity of the processes that require the many interrelated body systems to be functioning normally. We are making good progress in the development and 'characterisation of vessel on a chip' devices and their characterisation. Although these techniques have not yet reached sufficient maturity and

consistency for routine use, our current data suggest that this will be a realistic means to reduce, refine and replace the use of animal in platelet research in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

During this study we anticipate investigating the role of 10 proteins or processes in detail that are required for the control of platelet function. Five of these will be expected to require cell-specific (platelet/megakaryocyte) gene deletion, which required a larger breeding colony to obtained suitable genotypes and litter-mate controls. The numbers for maintaining strains are based on the use of published best practice guidelines for optimising colony size and using techniques such as cryopreservation of gametes and embryos where appropriate and possible. This will allow colonies to be maintained a suitable level when required to supply sufficient numbers of offspring for required experiments. Experiments will be designed to minimise animal numbers through the use of tools such as the Experimental Design Assistant (NC3Rs), power calculations and with experience of carrying out similar experiments under previous projects.

During the last project we have refined our scientific approaches to maximise the use of in-vitro and in- silico methods (as these have come available). This has allowed us to reduce the type and number of in-vitro animal studies that need to be done. In addition, we have optimised the number of animals needed for breeding and maintaining animal lines through further improvements in the health status of the colonies and archiving lines whenever possible. The latter has been done with facilities that allow access to these lines by other researchers in due course, and thereby reduce the necessity for others to re-create these lines.

Through this continuous application of the strategies to minimise the use of animals, we have been able to make a 50% reduction in use of animals from the previous project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

In most cases the levels of differences observed between different genotypes of mice within the context of the experimental approaches used are relatively high, and therefore it is possible to test hypotheses with small sample sizes. This is supported by power calculations for the specific experimental question and design and extensive experience in the use of the models proposed. Where new transgenic mouse lines are used we will

employ the Experimental Design Assistant developed by NC3Rs to formulate the most effective design which minimises animal use without compromising statistical power.

The following measures form routine components of our experimental design:

- Power calculations will be used to ascertain the minimum number of animals required to be used in a single experiment, to detect meaningful physiological effects. For the techniques within the project a power of 90% is realistic while minimising animal usage.

Mouse usage will also be minimised in the following ways:

- Use of efficient statistical designs to increase precision, e.g. use of litter mates as controls

- Use of efficient breeding protocols that minimise the production of unwanted animals

- Utilisation of heterozygous surplus mice to renew the breeding pairs used

- Use of modern efficient laboratory tests that allow fewer animals to be bred

- Researchers will be blinded to mouse genotype or treatment and animal selection randomised.

- We will seek statistical advice to help with experimental design and during data analysis to maximise the quality of data, while minimising variability. We have access to excellent statistical design and analysis expertise at this institution and within the applicant's BHF Programme Grant Team. Mathematical modelling for experimental design is also available within the applicant's research group.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Where new experiments are to be performed, and where new strains of mice are to be used, a pilot study will be conducted in accordance with N3CRs guidance (https://nc3rs.org.uk/conducting-pilot- study), in order the test the appropriateness of experimental design, the development of effective instructions to project researchers, determination of skills required and suitability of available equipment. This will enable the variability of results to be determined allowing power calculations to be performed for appropriate sample size determination, identify unexpected adverse effects and define early humane endpoints.

Experiments will be coordinated to ensure resources such as specialist analytical equipment are available to maximise use of samples and tissues. Where appropriate tissues will be frozen and stored for use in future studies or by others.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice (unaltered inbred strains and genetically altered mice) will be bred during this project. The offspring (and for unaltered inbred strain breeders at the end of their breeding life) will be used in protocols to assess the effects of genetic alterations on bleeding times, experimentally induced thrombosis and the modulation of platelet number. The effect of obesity and associated metabolic dysfunction on these outcome measures will also be assessed.

All mouse strains used in this project will either be transferred from the previous project or will be sourced from commercial suppliers or collaborators. We do not anticipate generating new lines under this project. As a result any adverse effects that may result from the genetic alteration will be well characterised. The majority of genetically altered mice used in our studies (and all mice used to date) exhibit no adverse effects, despite platelet function in some cases being altered substantially. This likely reflects the fail-safes that exist within blood clotting systems, to ensure that blood clotting occurs when it is really needed. In some cases removal of a gene systemically may be known to cause substantial harm or may be incompatible with development or survival. In these cases conditional deletion techniques are used to target deletion to the bone marrow cells that form platelets (megakaryocytes) and therefore also platelets, or other blood cells or blood vessel cells.

Mice will be used to assess bleeding and thrombosis, both of which will be performed under general anaesthesia. For analysis of thrombosis this will be without recovery, and for bleeding this will usually be without recovery. This ensures minimal levels of pain and suffering throughout these procedures. In some cases mice will be pre-treated with drugs or agents that either alter platelet function or the numbers of platelets in the circulation, before analysis of bleeding or thrombosis. All injections will be performed in accordance with minimal severity guidelines (e.g. Morton et al., 2001 Laboratory Animals, 35, 1-41). The injection should result in only transient pain and distress and no lasting harm.

Substances to be administered are not expected to cause pain or inflammation at the site of injection and any physiological effects are not expected to result in clinical signs. When a substance is to be administered to a particular strain for the first time, a literature search will be performed to see if information about the potential effects of the combination is available. Also a pilot study will be done so that any potentially significant adverse effects

can be assessed. These animals will be frequently monitored and those experiencing any unexpected adverse effect, will be killed humanely.

Finally, in some mice we will induce obesity through high calorie diet before mating and during pregnancy, while others will receive a normal diet to maintain normal weight and metabolic profile. Offspring from normal weight and obese mothers will also receive either normal or high calorie diet. A high calorie diet carries a low risk of causing a failure to thrive, and poor coat and skin conditions , although in our experience this is rare, and obese mice may display early stages of type 2 diabetes

(insulin resistance and elevated blood glucose levels). This approach has been refined in previous studies by colleagues, to minimise sample sizes and levels of suffering.

Why can't you use animals that are less sentient?

Similarities in the cell biology of platelets and the circulatory systems of humans and mice have led to mice becoming the non-primate model of choice in which to most closely replicate human blood clotting and thrombosis while also be amenable to the use of genetically altered mice. Less sentient animals such as zebrafish are used to study the molecular mechanism that control blood clotting, but it is not possible to study injury or vascular disease as would be experienced in the circulatory system of humans, and therefore for the study of bleeding and thrombosis a mammalian model is required. Our continued use of mice and refined experimental procedures will allow our work to continue to be set in the context of, and compared with, extensive literature of studies conducted using similar approaches, and to allow comparison with existing data from our own research.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our methodology will continue to be refined, with recent examples including new analysis techniques and statistical approaches that increase the information that may be gained from intravital microscopy analysis of thrombosis. This will include the continued development of the Virtual Platelet, and the establishment of more appropriate mouse models of platelet regulation that incorporates underlying insulin resistance that is present in many patients for whom anti-platelet therapy is required.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs website is used by my group as the primary source of published guidance which draws on extensive published academic studies and tools for appropriate experimental design. While we do look at primary literature to support for example refinement in techniques, the NC3Rs has compiled some excellent resources and continually updates and presents the latest evidence to support best practice. A good example of this is the Mouse Grimace Scale, and guidance on humane euthanasia which

are used in our work, and is supported by e-learning resources. Researchers will also refer to the PREPARE guidelines (https://norecopa.no/PREPARE) to assist in the preparation of new experiments and associated experimental design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will continue to use the NC3Rs website as a first source of information and training. This is an excellent resource in terms of training, best practice and emerging refinements that may be considered. Members of the group will be expected to attend webinars, including training in the use of the experimental design application and recordings of relevant previous sessions. These include efficient management of genetically altered mouse colonies, organ on a chip technology, the use of the PREPARE and ARRIVE guidelines 2.0, best practice in experimental design, and mouse handling made easy: reducing anxiety in mice and their handlers.

We have a learning platform (hosted on the Microsoft Teams platform) which is dedicated to dissemination of 3Rs-relative materials, advice and training and new developments to animal centre users, which is coordinated by a dedicated information officer and overseen by our veterinary surgeon. This is also used as a channel for information from the Home Office to users, and for sharing of good practice locally.

162. Molecular determinants of brain development

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

brain, development, neurogenesis, migration, circuits

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to understand the roles that different genes play during the development of the brain, both in normal conditions and in the case of neurodevelopmental disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The brain is the most complex organ in our body. It contains millions of neurons that establish trillions of contacts between them to ensure proper function. In addition, a similar amount of non-neuronal cells also play essential roles by supporting neuronal function in different ways. Putting together such a complex organ requires precise regulation of multiple processes. Many different genes are involved in the different stages, from the

production of the neurons to the recognition of the correct partners for circuit formation. Understanding how the brain is formed is important because defects during development have significant consequences on brain function that negatively impact the quality of life of those who suffer them and their families.

What outputs do you think you will see at the end of this project?

We expect to generate new information about the role of different genes during development of the brain, particularly of the cerebral cortex. One of those genes will be PCDH19, a gene mutated in a form of epileptic encephalopathy. We will publish our results in peer reviewed journals and present them in conferences in the form of posters or talks. The work conducted under this project licence will also be published in PhD theses.

Who or what will benefit from these outputs, and how?

Psychiatric and neurological diseases pose a heavy burden not only on society, but also on the health systems of many countries. The estimation of the European Brain Council as of 2010 was a yearly cost of €798 billion for the treatment of brain conditions, which represented 35% of Europe's total disease burden ("Cost of disorders of the brain in Europe 2010" (http://ebc- brussels.org/wpcontent/uploads/2015/07/Cost-of-Disorders-of-the-Brain-in-Europe-EurNeuro2011.pdf). Importantly, those numbers are now considered to be an underestimation and studies are ongoing to update them, exemplifying the importance of research into brain development and function.

The research proposed for this project comprises basic neurobiology, but it could also eventually be relevant from a clinical point of view in the future. The expected results would translate into two potential benefits:

First, it will generate new knowledge about the function of several genes, involved in distinct processes during cortical development, contributing to our understanding of brain development and function. This will benefit other researchers working on the field, but also clinicians dealing with patients affected by neurodevelopmental diseases, by providing new candidate genes for diagnostic screening.

Second, it will shed light on the pathophysiology of EIEE9, a human disorder caused by mutations in the PCDH19 gene (one of the genes that we will study), and potentially of other disorders, providing information to affected families to help them understand the disease.

A good understanding of the different molecules and processes involved in the development of the brain, specifically the cerebral cortex, is also essential when new risks are identified that can severely affect the mental health of the population (like the recent Zika virus epidemic). Such knowledge also forms the basis for the development of targeted interventions, in the form of drugs, behavioural, or others.

How will you look to maximise the outputs of this work?

There are several ongoing or planned collaborations that will help maximise the outputs of this work, including researchers at universities in the UK and Europe. The different collaborations focus on distinct aspects of the project, from neurogenesis and fate determination to circuit assembly and function, and we expect them to significantly enhance our outputs.

In addition, we will disseminate our findings through peer reviewed research papers in scientific journals and at meetings and conferences, some of them including clinicians and affected families (in the case of PCDH19). Furthermore, we will continue our ongoing involvement in public engagement, which focuses on children and young adults, mostly (but not exclusively) from disadvantaged backgrounds.

Species and numbers of animals expected to be used

- Mice: 3500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For this project the chosen species is the mouse. Most of our work focuses on the cerebral cortex, so we need to use a model animal with a cortical structure similar to that of humans, if we want to gain relevant insight that can be at least partially translated to human development. Mice are the lowest vertebrate group that has a six-layered cortex and are close enough to humans to reveal principles of brain development. Because mice have been extensively used as model animals in this field, there is a wide body of research to build upon and findings can be related to previous results.

A considerable part of our work is carried out in embryos, as this is the stage at which the processes of neurogenesis, fate determination and most of migration occur. However, to study the consequences that any alterations during development have on brain function, we need to use postnatal and adult animals.

Typically, what will be done to an animal used in your project?

The main techniques to be applied to the animals in this project are:

- breeding of genetically altered animals

- administration of substances by injection or in the drinking water: this can be done to a pregnant animal to have the substances reach the developing embryos, or to an adult animal to directly label specific cells. Injections to pregnant animals are a quick procedure; for adult animals they may require anaesthesia to direct the injections to specific location within the central nervous system.

- tissue collection for identification and analysis purposes: these are very short procedures. For analysis, they are carried out either after humane killing of the animal, or after terminal anaesthesia to the animal.

- in utero electroporation: this surgical procedure is performed on pregnant dams. It typically lasts less than 30 minutes and animals recover quickly after it.

- behavioural analysis: these procedures are mild and have minimal effects on the animals.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects that we expect are:

- transient increase in stress from behavioural analysis
- transient pain from tissue collection for identification/analysis
- postoperative discomfort
- very rarely post-operative complications

Most of the above-mentioned effects are expected to be of mild severity. Surgery is considered to have a moderate severity limit, but refinement methods should minimise any discomfort or pain to the animal.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect a majority of animals to be in the sub-threshold and mild categories. These are the animals that are used for maintaining the colonies, that only receive an intraperitoneal injection, or that are used in most behavioural analyses. Our experience from the last 6 years is that about 75% of animals fall in those two categories. An additional 10% are animals under the non-recovery category.

Regarding moderate severity, the proportion we expect is less than 10%. Those are mainly the pregnant animals undergoing surgery for in utero electroporation and adult animals that receive injections in the central nervous system.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The developing cerebral cortex is a very complex structure. It receives cells and signals that originate outside the cortex, and its cellular and spatial organisation, which is crucial for proper development, changes as development proceeds. At present, there are no non-animal models that can replicate these complex conditions. Also, to gain insight into the development of the human brain, a mammalian species needs to be used because lower vertebrates do not have a six-layered cortex.

Which non-animal alternatives did you consider for use in this project?

We have considered laboratory approaches which do not use live animals such as using mouse or human embryonic stem cells differentiated into neurons and the use of cerebral organoids.

Why were they not suitable?

The non-animal alternatives mentioned above will be used whenever a basic mechanism is studied that does not depend so heavily on the complex architectural and signalling arrangements of the live animal cortex. Mouse embryonic stem cells differentiated into neurons are already being used in the lab to study biochemical processes and neuronal responses. However, organoids still suffer from a lack of reproducibility and neither of the two alternatives allows for the study of behavioural changes resulting from developmental impairments.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This number has been estimated taking into account:

- the diversity of mouse lines needed to fulfil the objectives of the project
- the average number of animals used per year in the last 6 years
- the number of researchers in the group currently working with animals (2)

- the number of researchers that will join the group during the duration of the project (between 1 and 3)

- the ongoing and planned collaborations with other research groups

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

All calculations were made using the NC3Rs Experimental Design Assistant and current data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To ensure that animal numbers are kept to a minimum, besides experimental design, the following aspects will be considered:

1) Animal breeding will be optimised to reduce the number of surplus animals created that are not used in experiments. All researchers working on the project will watch the webinar “Best practices for efficient mouse colony management” that is available from the Jackson Laboratories. In addition, we perform a monthly audit of the mouse colony to ensure proper management.

2) Expression analysis of genes via publicly available databases and datasets prior to the start of any experiments will provide information about possible roles during development. Based on that information, experiments involving animals will be limited to meaningful hypothesis, i.e. if a specific protein is not expressed in progenitor cells, no experiments will be carried out to assess a role in neurogenesis.

3) Animal samples will be clearly labelled and properly stored to maximise its use through the course of the project. This is particularly relevant because analysis of developmental processes implies that experiments are required at multiple time points, including pre-natal stages. Because only whole litters can be harvested at embryonic stages, careful planning and consideration of future experiments will ensure that surplus samples are processed in ways that allow future use. This can also apply to animals that are used for behavioural experiments, which can then be perfused to generate a bank of tissue ready for further analysis.

4) Researchers will receive extensive training for the procedures where technical expertise significantly influences the success rate. The use of intrauterine manipulation techniques can in many cases eliminate the need to create knockout or knockin animals, thus reducing total animal numbers.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Choice of species and model:

To gain insight into the development of the mammalian isocortex, a species with a six-layered cortex needs to be used. For this project the chosen species is the mouse. Mice have an isocortex and are close enough to humans to reveal principles of brain development. Because mice have been extensively used as model animals in this field, there is a wide body of research to build upon and findings can be related to previous results. Most of the experiments will be carried out on wild type animals, but some genetically modified ones will be needed as well. They will primarily be already characterised reporter lines with sub-threshold to mild phenotypes. For knockout (KO) animals that might be imported into this licence, careful monitoring will be implemented to make sure that any adverse phenotypes do not exceed the severity limits.

Choice of methods:

The methods that will be used in this project include immunohistochemistry, in situ hybridisation, in utero manipulations, neuronal tracing and behavioural assessments. To maximise refinement, many of them are used ex vivo, on material obtained from animals that have been humanely killed. In vivo methodologies such as in utero manipulations will be used to address questions where the complex context of the developing cortex has to be preserved to obtain conclusive results. By affecting only a proportion of cells in the whole brain, in utero manipulations are more refined than conventional knockouts because the effect on the animal as a whole is reduced.

Surgery will be performed under general anaesthesia and under aseptic conditions to prevent any post- surgical infections. Analgesics will be administered as standard to minimise suffering, and animals will be monitored regularly to ensure their wellbeing.

Training of these procedures is done in a very gradual step by step way, due to the technical skills needed. No greater loss is expected during training of new licensees / members of the group. Training for intracranial injection into early post-natal animals will be obtained from other groups performing this technique at the home establishment, or from collaborators in other universities. No injections will be performed until the licensees are deemed competent by an experienced trainer.

Why can't you use animals that are less sentient?

The six-layered cortex (or isocortex) is present only in mammals, so invertebrates like the fruit fly or lower vertebrates like the zebra fish, although useful for some studies (genetic interactions, cascades), cannot be used in this project. Mice are the lowest vertebrate group in which the experiments required to complete the project can be performed. They have an isocortex and their patterns of cortical development are similar to those in primates. Despite a much smaller size and a lower complexity, much of what we know about cortical development in humans has been contributed by studies carried out in mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Whenever we receive or generate new strains of genetically altered animals, we will monitor them closely, in collaboration with the animal technicians. If any adverse effects become apparent, we will seek the help of the named veterinary surgeon to assess the severity of the phenotype and discuss how to adjust our analysis to avoid animal suffering. This might include restricting the analysis to earlier time points (before the appearance of symptoms, or before the symptoms worsen), using mutants in which the gene has been deleted only in specific tissues, or using techniques (such as in utero electroporation) to reduce the number of neurons affected and, therefore, any assocaited negative effects.

Intrauterine surgery will be performed under general anaesthesia in a form and dose suitable for the species and under aseptic conditions to prevent any post-surgical infections. Analgesics suitable for the species will be applied peri-operatively as standard to minimise suffering, and animals will be monitored regularly (at least twice on the day of surgery, then once daily until the end of experiment). A general pain-scoring chart will be used to record the well being of the animals, as well as any adverse effects specific to the operations performed on that animal. To reduce the stress levels of animals used in behavioural analysis, they will be handled prior to the experiment, so that they can get used to the researcher.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow the guidelines published by the N3CRs and any other refinement measures that are published in the literature (particularly in methods papers). Our collaborators are also very experienced and have excellent track records in their respective areas, being able to provide further guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive the monthly newsletter from the N3CRs by email, allowing us to stay on top of advances in the 3Rs. We will also attend local and regional 3Rs symposia and keep our

communication with the animal technicians and NACWOs, who can be very helpful, especially with the implementation of refinement advances.

163. Neuronal and glial function and dysfunction across the life-course

Project duration

5 years 0 months

Project purpose

Basic research
Key words

brain, neurodevelopmental disorders, neurodegeneration, neuron, glia

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Our overarching aim of our programme of research is to determine how cell to cell communication in the brain is altered in diseases or disorders which affect either its normal development or cause its degeneration.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

An estimate from 2010 indicated that the financial burden in the UK of brain disorders (neurodegenerative and neurodevelopmental diseases, psychotic disorders, clinical

depression, anxiety and addiction) was around £70billion. Fundamental research into the mechanisms of brain disorders of the sort we conduct are the first step in understanding how they can be either prevented or treated. Given the ability to mimic the genetic bases in rodents of many of the disorders we study this gives us hugely valuable information about the origins of disorders, whether they show common traits in terms of the dysfunction they cause and whether interventions either genetic or pharmacological can ameliorate the dysfunction that is present. This Licence seeks authority to conduct experiments which are aimed at increasing fundamental knowledge about ‘normal’ neuronal and glial function and how such function is altered in genetically altered animals (GAAs) that we use to model neurodevelopmental and neurodegenerative (including excitotoxic-induced) diseases (NNDs) and complements work we also do using human pluripotent derived neurons and glia to understand these disorders.

What outputs do you think you will see at the end of this project?

The major form of output from this work will be publications in peer-reviewed scientific journals. Since 2016 the individuals named in this application have published 77 papers; together these outputs have already been cited more than 1000 times. Indeed the 10 publications that are co-authored by each of the named individuals account for more than 25% of all citations reinforcing the notion that our individual expertise when combined gives rise to impactful science. In addition to primary research papers, authoritative reviews will be published and we will continue to be (co)-authors on position statement/papers that influence the research field within which our work contributes.

Who or what will benefit from these outputs, and how?

The major beneficiaries of our research will be colleagues working in our and related-research areas. They will be informed of our findings primarily through our publications. Some of our research may offer the potential identification of novel therapeutic targets and thus would contribute to the first steps of the drug discovery process for the pharmaceutical industry. A longer term benefit will be the contribution of fundamental knowledge as to the mechanistic bases of the diseases and disorders we model. Arguably this offers hope to patients and their families that therapeutic interventions may be possible in the future given the huge clinical unmet need that currently exists.

How will you look to maximise the outputs of this work?

We publish our work in leading international scientific journals, ensuring that our local Press Office is aware of publications that are likely to have significant impact or are of interest to the general public, patients/patient families. In addition we highlight our work through Public Engagement activities at science festivals, patient support groups and other stakeholder events. Where possible we publish our papers to allow immediate Open Access (Gold) to allow anyone in the world to read about our findings. Complete datasets are available to researchers on reasonable request.

Species and numbers of animals expected to be used

- Mice: 14500
- Rats: 29000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Invertebrate model organisms have been used widely in the field of synaptic neurophysiology and while studies using such organisms inform our work the focus of our research requires mammalian central nervous system (CNS) tissue where the complement of neurotransmitters, their receptors and the overall architecture/structural units/function of the nervous system is closely related to that of humans. Rodents are the most appropriate species for our work not only because they are amenable to genetic manipulation but studies have identified that the key proteins that will be the subject of investigation are found in both rodents and humans. Our research programme spans disorders that affect the developing brain and the aged brain and as such we need to use animals of an appropriate age that reflects the disorder under investigation.

Typically, what will be done to an animal used in your project?

The vast majority of animals will be bred so that we are able to collect brain tissue from appropriately aged animals for subsequent ex vivo electrophysiological recording, biochemical or neuro-anatomical studies. In addition, some animals will be terminally anesthetized for prolonged periods to assess the impact of reduced brain activity on cellular communication as assessed subsequently by ex vivo electrophysiological recording, biochemical or neuro-anatomical investigations. A small proportion (25%) of animals will be allowed to recover for up to 72 hours prior to culling for subsequent ex vivo studies. Furthermore to assess the effects of increased brain activity some animals will be terminally anesthetized but prior to culling will subjected to non-invasive transcranial brain stimulation; to allow analysis of longer-term effects of electrical stimulation on CNS tissue, a small proportion (25%) will be allowed to recover from anaesthesia for subsequent culling up to 72 hours later. Related to decreased brain activity, some animals will be dark-reared so that they do not have visual experience in order that we can assess the impact of this loss of cellular communication in the neurodevelopmental

disorders we study. Seizures are a very common feature that humans with neurodevelopmental disorders experience and we will assess seizure susceptibility in our rodent models by exposing them to loud noises. Finally, the Licence will allow us to administer substances that would be expected to alter cellular communication or we will re-activate disease-causing genes whose activity has been previously 'silenced' to assess the potential for either reversal or amelioration of pathophysiologies caused by loss of specific gene function.

What are the expected impacts and/or adverse effects for the animals during your project?

Some animals may have the potential to develop a harmful phenotype, eg tumours, neurological signs, after a certain age but in the majority cases will be killed before reaching that age and if they do reach this stage they will be monitored daily to determine whether the defined humane end points have been reached. Some animals may occasionally exhibit absence-like seizures. These are not considered to be detrimental to the health of the animal. Animals with altered immune status will be maintained in a barrier environment thereby minimising the likelihood of compromising health. Daily monitoring and appropriate animal husbandry will identify any animal experiencing detrimental impact during housing. Protocols in this PPL are designed to minimise all adverse impacts.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals (both mice and rats) will experience only mild severity impacts - these will account for approximately 70-80% of all animals. The remaining animals (both mice and rats) will experience moderate severity impacts. No protocol on this Licence has a "severe" banding.

What will happen to animals at the end of this project?

Killed
Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Humans share >97% genetic identity with mice and rats and as such these rodents are models with which we can carry out experiments to understand how the brain works. Non-animal alternatives are extremely useful to getting us “part of the way” but ultimately we need to study the mammalian brain and mice and rats are the lowest order mammal in which this can be done reproducibly while having the ability to perform genetic manipulations that allow us to create structural validity of disease.

Which non-animal alternatives did you consider for use in this project?

Complementary research in our lab uses systems which allow us to express, in cell culture systems, proteins that may be affected in neurodevelopmental and neurodegenerative disorders. Such studies are extremely valuable in assessing the properties of these

proteins. Moreover, we make use of technologies that allow us to generate human neurons and glial cells from human skin cells - so-called induced pluripotent stem cell models. This research complements our work with rodent model systems.

Why were they not suitable?

Cell culture systems do not reproduce the exquisite cellular architecture nor all the different cell populations that exist in the brain. In addition, cell culture systems do not allow us to reproduce the circuit networks found in the brain that are so crucial for brain signalling.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

There are a large number of PIL holders (approximately 30) who work under the authority of my current PPL. The numbers estimated are based on my Annual Return over the past 5 years. For a given PIL holder using five animals per week, over an average of 40 experimental week in a year, this gives an average of 200 animals. Over five years, this equals 1000 animals and with 30 PIL holders then a total of approximately 30,000 animals. Our use of rats and mice depends on the nature of the experiment to be conducted but over the past five years we have used these in a ratio of 2:1 (rats to mice), hence giving the estimated number indicated above.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

It is essential that the number of animals we use allows us to collect data that are robust. We have many years' of experience in performing experiments using animal tissue and this allows us to make predictions, using statistical analysis based on previous data and the expectation of effect size, about the anticipated effect size we are likely to see and how variable that effect will be. Using this information we can estimate the numbers of experiments we need to carry out. Our experience indicates that for the effect sizes we anticipate we require between 8 – 10 animals to ensure statistical robustness. Good experimental design means that we assess drug effects alongside non-drug treatments or control tissue with that obtained from genetically modified animals. All PIL holders working under the authority of this PPL will be asked to take the local Research Optimisation Course that covers rigorous experimental design, research conduct, analysis and the reporting of animals research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We encourage, where possible, that experimenters share tissue from the same animal which will reduce the overall number of animals required for experimentation. We seek advice from statisticians where appropriate when planning experimental designs.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice, together with rats, represent the most commonly used mammal to study central nervous system function. While genetically-altered mice have been models of choice for more than two decades, recent advances in transgenic technologies, together with the larger brain size of the rat and the increased behavioural repertoire they display means that is it now possible to conduct studies of brain signalling and its dysfunction. Such studies allow for cross-species comparisons which is essential when one considers the potential for translating findings from 'basic' research to potential therapeutics. The majority of the GAAs we study display few visible signs in terms of alterations in their physical features, development, behaviour or their ability to thrive. Indeed it is only with sort of detailed experiments we propose to carry out that deficits in brain signalling are revealed. Some of the pre- clinical models we use are classified as have a "moderate" phenotype as the mutation has the potential to cause harm if the animal was to live for a long time. However, we closely monitor our GAAs and if they show signs of distress they are killed humanely. For our experiments we use animals before these phenotypes develop. The animals in these studies will be cared for by trained staff within a well-resourced and well-equipped modern animal facility that contains individually ventilated cages (IVCs) and barrier systems to maintain specific pathogen-free (SPF) status/health. The majority of work to be conducted under this PPL will involve humanely killing animals to obtain tissue for subsequent experiments that examining the electrical activity of neurons and glial, or the biochemical signalling and its control that occurs within cells. Such experiments are all performed ex vivo. Some protocols involve exposing them to loud noises to assess seizure susceptibility, anesthetizing animals for prolonged periods or rearing in a dark environment. In the case of the latter two methods these are not considered to cause lasting harm. In the case of exposure to loud noise we need to use this method as it attempts to mimic the increased sensitivity some people with

neurodevelopmental disorders have to sensory inputs. This is a non-invasive method of assessing susceptibility and all animals experiencing this are culled immediately after exposure.

Why can’t you use animals that are less sentient?

The brain disorders we study affect humans and as such we need to model these in a mammalian system. Rodents such as mice and rats are the lowest order of species that are sufficiently genetically related (97%) to human to allow us to obtain data where we can begin to understand what goes wrong with cellular communication in people that suffer from these disorders. The majority of our work involves the collection of brain tissue from terminally anesthetized animals and which we use ex vivo to understand the mechanism by which dysfunction is manifested.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As required our rodent colonies are monitored daily for their welfare. As noted in the application most of the animal models we study display mild cognitive and behavioural deficits. Where a mutation/deletion has the potential to cause a harmful phenotype, animals are culled before these manifest. For animals where we administer substances, they are monitored frequently to check for any adverse reactions and if these are observed we take appropriate action or seek veterinary advice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will consult and refer to publications and resources produced by NC3Rs (www.nc3rs.org.uk). These include advice on Experimental Design and 3Rs Self-assessment tools to maintain oversight of our use of the most refined protocols. Where appropriate we will consult PREPARE guidelines and ensure that we follow best-practice, veterinary advice to ensure most appropriate routes for substance administration and induction/maintenance of general anesthesia

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are informed of the latest findings and proposals from organisations such as National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and Understanding Animal Research (UAR). We discuss these within our research group and consult with veterinary staff to ensure that, where appropriate for our research programme these are implemented.

164. Understanding the mechanisms of the carotid body hyperactivity caused by chronic hypoxia

Project duration

3 years 0 months

Project purpose

- (a) Basic research

Key words

breathing problems, low blood oxygen, carotid body, high blood pressure, irregular heart beat

Animal types	Life stages
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to identify how a persistent fall in blood oxygen (called hypoxia) causes hyperactivity of the carotid body- the organ in the body which constantly senses and responds to changes in the blood oxygen. The project will also look at how this change in carotid body function leads to alterations in breathing, heart function and blood pressure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The carotid body is an important organ located in the neck that constantly monitors the blood supplying the brain. When the blood oxygen level drops (called hypoxia), the carotid

body immediately triggers an alarm in the form of thousands of nerve impulses sent to the brain. This activates important reflexes including an increase in breathing and heart rate, and narrowing of most blood vessels, so that enough blood and oxygen can instead be delivered to the brain. However, in patients with irreversible lung disease (such as chronic obstructive pulmonary disease) the blood oxygen level is always too low. The carotid body is constantly stimulated and over time becomes hyperactive. The hyperactive carotid body causes persistent over-stimulation of the cardiovascular/respiratory reflexes leading to a sustained rise in the nerve activity going to the blood vessels and heart. These patients therefore develop a high blood pressure and an irregular heartbeat. However, the mechanism of carotid body hyperactivity caused by lung disease and sustained hypoxia is poorly understood and currently there are no treatments used clinically that directly target the carotid body. A better understanding of the mechanisms underpinning carotid body hyperactivity and the rise in cardiovascular/breathing reflexes in response to chronic hypoxia would be an important first step towards developing new treatments for carotid body mediated cardiovascular disease.

What outputs do you think you will see at the end of this project?

This project will provide new information about the underlying processes that lead to carotid body hyperactivity in response to a persistent fall in blood oxygen (hypoxia). In addition, the project will reveal how this change in carotid body function can lead to alterations in heart function, breathing and blood pressure. This information is likely to be published as scientific articles in high impact cardiovascular and respiratory journals.

Who or what will benefit from these outputs, and how?

During and by the end of the PPL, the findings will greatly advance the biological understanding of how prolonged exposure to low blood oxygen causes the carotid body to become hyperactive. The project will show how the overactivity of the carotid body leads to important downstream changes in breathing, heart function and blood pressure. This new knowledge will be of benefit to scientists and clinicians who work on understanding the disease processes in the carotid body, heart, lungs and blood vessels. These findings will also benefit scientists and clinicians who work more broadly on understanding how exposure to low oxygen leads to disease. The new information should be an important first step in moving towards developing future treatments for carotid body mediated disease. The new information provided by the end of this project will therefore be beneficial to biotechnology companies that are aiming to develop new treatments associated with lung disease in which patients have a persistently low blood oxygen.

How will you look to maximise the outputs of this work?

We will disseminate important research findings at the most prestigious cardiovascular and respiratory conferences across the world. Furthermore, we will publish the findings in the form of research articles in internationally recognised cardiovascular and respiratory journals. We will aim to publish articles based on work in this PPL as Open Access to

extend reach, influence, and impact. Negative findings, unsuccessful approaches and null data will still be published in journals which encourage reporting of all data. I will also disseminate our research to the general public by hosting lab tours and seminars organised by our funders. Whilst talking about animal experimentation can be a contentious subject, I do believe that this provides an excellent opportunity to discuss the 3R principle and scientific studies involving animals in the UK to broaden perspective and challenge preconceptions.

Species and numbers of animals expected to be used

- Rats: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

High blood pressure and irregular heartbeats caused by exposure to low blood oxygen develop as a consequence of alterations in whole body reflexes pathways. This includes a persistent rise in nerve activity to the heart and blood vessels triggered by carotid body hyperactivity. This experimental programme is therefore centred on integrative whole-body reflex responses and requires in vivo research.

Juvenile and young adult rats will be used for this project once carotid body function is fully developed. Importantly, rat carotid body, cardiovascular and breathing function is sufficiently close to that of humans to allow transfer of findings to humans. For example, rats exhibit carotid body hyperactivity, high blood pressure and a high heart rate in response to persistent falls in their blood oxygen. These are the same changes seen in human patients with lung disease such as chronic obstructive pulmonary disease. We will use rat rather than mouse, as the reflex responses to low blood oxygen in the rat are more similar to humans and take place at more comparable oxygen levels. The reflex responses to low blood oxygen in the rat are also better maintained than in the mouse, again showing more similarity to humans. Thus, the rat offers a better model of disease relevant to humans for the specific aims of this project. Using the rat will therefore provide more valuable data in terms of promoting future translation to human disease. Searches for alternatives to in vivo (e.g. NC3Rs, NORINA) integrative experiments in this field do not provide replacements for the animal experiments proposed in this project.

Carotid body function is not fully developed in the womb or immediately after birth. Therefore, we will use animals at a minimum of 5 weeks or older once the carotid body has fully developed and is fully functional.

Typically, what will be done to an animal used in your project?

Some animals will be housed in normal ambient oxygen (approximately 21% $O2$,) throughout and then have carotid bodies isolated under non-recovery terminal general anaesthesia with killing completed by a schedule 1 method.

Some animals will breathe a lower environmental oxygen (by introducing a lower environmental oxygen mixture into a environmental chamber, 10-12% $O2$), for up to 10 days. This level (10-12% $O2$) and duration (up to 10 days) of lower environmental oxygen is chosen based on published evidence shown to cause long-term changes in carotid body function in rats. These animals will then have carotid bodies isolated under non-recovery terminal general anaesthesia with killing completed by a schedule 1 method.

Some animals will be housed in either normal ambient oxygen (approximately 21% $O2$) or a lower environmental oxygen (10-12% $O2$) for up to 10 days (approximately equal numbers per group). Acute breathing measurements will be measured up to two times per animal using non-invasive, whole body plethysmography. During this procedure, animals will be placed in the plethysmography chamber with a reduced floor space for a maximum of 3 hours. A rise in breathing is measured in response to short durations (maximum of 5 minutes) of low environmental oxygen (minimum 8% $O2$) and higher carbon dioxide (6% $CO2$). After these breathing reflexes have been made, animals will be returned to their home cage. A single injection of a substance aiming to lower carotid body activity (or control substance) may be administered before the second set of breathing measurements are made. Any single injection will be given based on known routes, doses and volumes published in the literature and will not exceed the maximum volume defined by the LASA guidelines. Animals will then be killed by a schedule 1 method or in vivo measurements of blood pressure and heart function will be made under non-recovery terminal general anaesthesia with killing completing by a schedule 1 method or carotid bodies will be isolated under non-recovery terminal general anaesthesia with killing completed by a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

When the animals are placed in a lower environmental oxygen (10-12% $O2$) environment they are expected to demonstrate an increase in breathing rate and at times there will be noticeable larger breaths (sighs). The animals may also appear lethargic and it is expected that the activity of the animals will decrease especially on the first day. It is also likely that the animals will not have the same appetite and are expected to eat slightly less than usual especially during the first day. However, it is expected that the animals will adapt well and whilst the increase in breathing will continue throughout the duration of exposure to the lower oxygen environment, the animals will resume normal exploratory and feeding behaviour within 1-3 days.

When making acute measurements of breathing reflexes using non-invasive, whole animal plethysmography, animals will be placed in a plethysmography chamber with a reduced floor space of approximately 280 cm2. This will be temporary (up to 3 hours). The animals

are expected to explore around the plethysmography chamber as they get used to the new environment. When undergoing the short (up to 5 minute) exposure to environmental low oxygen, the animals will demonstrate an elevated breathing rate, some instances of larger breaths (sighs) and a reduction in activity. When undergoing the short (up to 5 minute) exposure to higher environmental CO2, the animals will demonstrate an elevated breathing rate, some instances of larger breaths (sighs) and an increase in exploratory and grooming behaviour. The administration of substances before the second measurement of breathing will be undertaken using a combination of doses, volumes and routes that of themselves will result in no more than transient discomfort and no lasting harm.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Approximately a third of animals will undergo induction of anaesthesia only one one occasion; those that are housed in normal ambient oxygen and and then have carotid bodies isolated under non-recovery terminal general anaesthesia with killing completing by a schedule 1 method.

Approximately two thirds of animals will experience procedures which fall under the moderate severity including either exposure to environmental chronic hypoxia using an adjustable environmental chamber and/or measuring acute breathing reflexes using whole body plethysmography.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The carotid body (CB) is the key oxygen sensor in the body that communicates with the brain to alter nerve activity to the heart, blood vessels and breathing muscles. However, in response to a long term fall in blood oxygen, the carotid body becomes hyperactive which causes a persistent rise in the reflex pathways and the nerve activity to the heart, blood vessels and breathing muscles. This research programme is centred on whole body reflex responses involving communication between multiple organs and therefore requires in vivo research. It is not possible to answer the key questions without using animals. Searches for alternatives to in vivo (e.g. NC3Rs, NORINA) integrative experiments in this field do not provide replacements for the animal experiments proposed in this project.

Which non-animal alternatives did you consider for use in this project?

Searches for alternatives (eg NC3Rs, NORINA) to integrative experiments in this field do not provide replacements for the animal research proposed in this licence. If we find that technology advances during the lifetime of this project, we will not hesitate in using non-animal systems if they are suitable to address our scientific question.

We have considered using culture based neuronal cell lines e.g. PC12 cells to identify the local effects of hypoxia. However, this does not allow us to investigate changes in reflex pathways in the whole organism.

Why were they not suitable?

There are significant limitations of basic culture conditions using cells gown in isolation and this is not an accurate representation of what occurs within a whole organism. A reason why many new drugs fail between cell culture and in vivo studies is in the inability to fully recapitulate the in vivo environment.

This experimental programme is centred on integrative whole body cardiovascular/respiratory reflex responses arising from carotid body stimulation, leading to downstream changes in breathing, heart and blood vessel function. Using cell lines would not allow us to investigate the pathological changes in carotid body function on the cardiovascular/respiratory reflex pathways in the whole organism. As such, it is not possible to answer the key questions using just cell lines and without using animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers have been estimated by calculations that are based on our own pilot data, previous in- house data and published studies by ourselves and others. Experimental design has been discussed with, and approved by, our statistical advisor and further approved by both internal and external peer review by our funders. These estimates will be updated and recalculated throughout the project as we generate new data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have taken a number of steps to ensure animal numbers are reduced and that experimental data is reliable and reproducible:

Several procedures will be performed using a single animal where this does not unduly increase the burden on the individual animal (e.g. non-invasive breathing measurements, followed by measuring cardiovascular reflexes and heart function under non-recovery terminal general anaesthesia).

Animals will be randomly assigned into experimental groups.

Rats in the control and experimental groups will be of a similar age and the same strain to reduce variability in age and genetics.

Control and experimental groups will have the same diet and access to water ad libitum. This will reduce variability due to diet.

Non-invasive in vivo breathing measurements will be made on control and experimental

rats at the same time of day to reduce variability due to changes in circadian (daily) rhythms.

All in vivo and ex vivo data will be presented as mean per animal data, to avoid technical replicates.

Where possible, experiments and data analysis will be performed blind to to whether or not animals have been exposed to a low environmental oxygen, to minimise investigator bias.

We will use well established experimental methods to reduce variability

We will use appropriate n numbers based on power calculations performed before the experiment has begun, this will ensure that we do not use too many animals.

We will optimise certain ex vivo techniques e.g. antibody concentrations, on cell lines prior to use of tissue/cells isolated from animals

All experiments have been designed in accordance with the NC3Rs Experimental design assistant.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Both carotid bodies will be removed from each rat to allow for use in multiple ex vivo assays. E.g.1 carotid body could be used for analysis of carotid body nerve activity and the other used for analysis of gene expression.

If we are unable to estimate an effect size from our in vitro data, the literature, or our collaborators, we will conduct small pilot experiments.

Where possible, tissue surplus to requirement will be shared post-mortem for other ex vivo experimentation.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

<u>Exposure to low environmental oxygen using an adjustable environmental chamber.</u>

This procedure is based on peer review and is a well-established method. The model is non-invasive and is achieved by reducing the O2 delivered to the adjustable environmental chamber to achieve an environmental O2 of 10-12%, for up to 10 days. To aid with animal adaptation, on the first day, the O2 will be gradually lowered over a period of hours before reaching the final value of 10-12% O2. The animals will be closely monitored during this adaptation period and for the first hour at 10-12% O2. This level (10-12% O2) and duration (up to 10 days) of low environmental oxygen is chosen based on published evidence shown to cause long-term changes in carotid body function and long-term increases in cardiovascular/breathing reflexes in rats. Breathing this level of oxygen (10-12% O2) also leads to a blood oxygen level similar to that seen in patients with lung diseases such as COPD. On the first day of low environmental oxygen exposure, the animals will demonstrate an increase in breathing, a slight decrease in activity and a slight reduction in feeding. However, it is expected that the vast majority of animals (>99%) will adapt well and, whilst the increase in breathing rate will continue, the animals will resume normal exploratory and feeding behaviour within 1-3 days. This method has been used successfully for many years by our collaborators at other institutions and is based on published work. The animals are transferred to a cage that has bedding and enrichment objects from the animal’s home cage to reduce stress. Animals are unrestrained and food and water is available ad libitum. To monitor animal welfare, animals will be briefly removed from the environmental chamber for measurement of body condition score and weight loss. This will be done a minimum of once per week with routine visual checks performed daily or more often as needed.

This is the least invasive method of causing a persistent fall in blood oxygen in animals. There are other models based around severe irreversible lung damage that we have ruled out. These include the injection of a enzymes into the lung to evoke significant long term damage and the use of virus-induced lung injury. These models that we have ruled out are significantly more severe and cause the animals increased amounts of long term stress,

suffering and can potentially be lethal. Furthermore, these methods both lead to strong immune responses making it difficult to pinpoint the specific effects of the low oxygen on the carotid body. Another method that causes a long term fall in blood oxygen is a complex recovery surgery to induce chronic heart failure. Whilst this procedure is shorter in duration, it requires exposure to general anaesthesia and a period of mechanical ventilation. Thus, it is more invasive, will cause more stress to the animal and more long-lasting harm. Also, in this instance any heartbeat irregularities might be a consequence of direct damage to the heart rather than the impact of the increased nerve activity to the heart. Thus, our proposed model is the least invasive but still causes relevant changes in carotid body function and cardiovascular/respiratory reflexes that are required to achieve the aims of the project.

Non-invasive unrestrained whole body plethysmography (WBP)

Measurements of breathing will be performed on awake animals using non-invasive unrestrained whole body plethysmography. This is the least invasive method to record breathing in awake animals. The maximum time animals have reduced floor space (approximately 280 cm2), is 3 hours, but are not restrained. Animal bedding from the home cage will be added to reduce animal stress. The technique allows for breathing to be measured without the potentially confounding effects of anaesthesia or the stress of restraint. The levels of acute lower oxygen (minimum of 8% O2) and higher carbon dioxide (maximum of 6% CO2) used to activate the carotid body and increase the breathing have been chosen based on published literature and are known to cause moderate carotid body stimulation without initiating defence responses, vocalisation or escape behaviours. The duration of individual episodes of lower oxygen or higher carbon dioxide or both is short but is long enough to measure the steady state rise in breathing. When undergoing exposure to lower oxygen or higher carbon dioxide or both, the animals are likely to demonstrate an increase in breathing and some instances of larger breaths (sighs). Animals may also demonstrate either a reduction in activity (during low oxygen) or an increase in activity and exploratory behaviour (during higher carbon dioxide). These effects are short lasting and are quickly reversed after switching back to normal oxygen and carbon dioxide. Measuring breathing in this way is expected to cause no lasting harm.

Measurements of cardiovascular reflexes and cardiac arrhythmia induction under non-recovery terminal general anaesthesia

Cardiovascular reflexes (blood pressure, blood flow) and heart electrical activity (ECG, heart rhythm, heart rate) will be measured in animals under non-recovery terminal general anaesthesia. The procedure requires surgical tracheal and blood vessel cannulation as well as pacing the heart with an electrode. The procedure will assess the electrical integrity of the heart by pacing the heart at high rates. Measuring these parameters in the whole animal allows for the nerve communication between the different organs to be maintained (e.g nerve activity to the blood vessels and heart that is controlled by the carotid body and the brain). Whilst there will be effects of the anaesthetic, we will minimise this by selecting agents that achieve surgical anaesthesia but have minimal direct effects on the carotid

body, cardiovascular and respiratory systems, as previously published by ourselves and others.

Sufficient surgical anaesthesia will be assessed by absence of a pedal withdrawal reflex and monitoring of breathing rate. The maximum time under general anaesthesia will be 4 hours and following experimentation animals will be euthanised by a Schedule 1 method.

Carotid body isolation under terminal non-recovery general anaesthesia

The carotid body is excised under deep non-recovery terminal general anaesthesia. The carotid body is removed from the animal whilst still being perfused in order to limit the extent of metabolic insult, to ensure the most valid and reproducible ex vivo measurements of carotid body function are generated.

Sufficient surgical anaesthesia will be assessed by absence of a pedal withdrawal reflex and monitoring of breathing rate. The maximum time under general anaesthesia is 45 minutes. Following carotid body removal, animals will be euthanised by a Schedule 1 method.

Why can’t you use animals that are less sentient?

The carotid bodies and reflex responses to low blood oxygen are not fully developed in rats until at least 2-4 weeks of age. We cannot use embryonic forms as the carotid body is not yet fully formed/functional. We will therefore study carotid body hyperactivity and cardiovascular parameters in rats when animals are of an age where the carotid bodies and cardiovascular reflexes are fully developed. We have chosen to use rats over other less sentient species such as Danio Rerio (zebra fish) and drosophila melanogaster (the fruitfly) as these animals do not have similarly developed carotid bodies and whole body cardiovascular and breathing reflexes in response to low oxygen and highercarbon dioxide. Importantly, rat cardiovascular and breathing reflexes are sufficiently close to that of humans to allow transfer of findings to humans. We will use rat rather than mouse, as the reflex responses to low oxygen in the rat are more similar to humans and take place at more comparable oxygen levels. The reflex responses to low oxygen in rats are also better maintained than in mice, again showing more similarity to humans. Thus, the rat offers a better model of disease relevant to humans for the specific aims of this project. Where possible we will obtain data under terminal non- recovery general anaesthesia. For example measurements of acute cardiovascular reflexes and heart function will be performed under terminal non-recovery general anaesthesia.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

At the end of each experiment, we will continue to undertake a critical review to see how it can be refined going forward.

Over the years we have already implemented a number of refinements in regards to the use of the adjustable environmental chambers. For instance, to aid with animal adaptation, on the first day, the oxygen will be gradually lowered over a period of hours before reaching the final value of 10-12% O2. During this time the animals are closely monitored. After reaching the 10-12% level of oxygen, the animals will be closely monitored for the first hour and in the first 1-3 days of exposure, when animals are most likely to demonstrate reduced activity and increased breathing frequency, will we perform more frequent visual monitoring. After the first 3 days, routine visual checks will be performed daily or more often as needed. We will perform specific welfare checks including body-condition scoring and measurements of weight loss on a minimum of one occasion per week. Whilst in the environmental chamber, animals are housed in same-sex pairs rather than individually to minimise social stress. We also add in bedding and enrichment objects from the animal's home cage to reduce stress. All of these refinements will be implemented in this PPL. Furthermore, we will continue to review the technique to see how it can be further refined in the future.

For the non-invasive breathing measurements we have also already implemented changes to refine the technique and reduce animal harm. For instance, animals have at least 2 short visits to the recording chamber to get used to the new environment before the actual experimental procedure. Furthermore, we add in bedding into the plethysmography recording chamber to help adapt to the different environment and reduce stress. These refinements will be implemented in this PPL. We will also continue to review the techniques to see how they can be further refined in the future.

We will introduce a programme of acclimatisation where the user will increase the frequency of handling before entering onto the protocol. This will include rat tickling. This helps improve the rat- handler relationship and decreases animal stress. We will also monitor best practice guidelines for rat handling and implement any suggested changes that emerge in the future.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

I will use NC3Rs website for information and follow LASA guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will aim attend workshops hosted by the NC3Rs. In addition, my lab will comply with the ARRIVE guidelines (Animal Research: Reporting In Vivo Experiments; www.nc3rs.org.uk/arrive), a NC3Rs- developed checklist of the essential information that should be included in publications reporting animal research. This will include reporting of the essential 10:

1) type of study design and how controls were defined

2) total number of animals used, sample size and what was classed as a single experimental subject for each experiment

3) inclusion and exclusion criteria

4) if and how animals were randomly assigned into each control and experimental group

5) whether the investigator was blinded during the acquisition and analysis of data

6) outcome measures and if (and why) any have not been included in the article

7) full descriptions of data analysis and statistical methods used

8) species, strain, substrain, sex, weight, and age of animals

9) essential information relating to the experimental procedures performed

10) summary of descriptive statistics for each experiment including some measure of variability (e.g. SE, SD, interquartile range), and where appropriate the effect size and if we consider this to be of biological relevance

Myself and team members will also sign up to receive the NC3Rs newsletter.

I will also review literature regarding the specific procedures that we perform to see if they can be refined further.

Any new advancements will be made clear to members of my team through our weekly lab

165. Regulation of immune responses in cancer

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immune responses, Lymphocytes, Immunotherapy, Metabolism

Animal types	Life stages
Mice	pregnant, adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project will study immune responses to tumours with an overall aim of identifying new approaches for the treatment of cancer. We are particularly interested in understanding how responses of populations of white blood cells called T lymphocytes are turned on or switched off in response to tumours sited in distinct areas of the body and the role of metabolism in these processes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Despite advances in the treatment of cancer, the World Health Organisation reports that cancer remains the second leading cause of death worldwide. In recent years, novel

approaches that harness the body's own immune defences against tumours, collectively termed immunotherapies, have proven highly effective in the treatment of otherwise incurable cancers such as malignant melanoma, a form of skin cancer. Nonetheless, for several cancer types these approaches have yet to bring benefit to patients, whilst even with "immunotherapy-sensitive" cancers, a proportion of patients do not respond. Therefore, there remains an urgent need to understand better how immune cells respond to tumours and the mechanisms that tumours exert to prevent effective immune responses. This work will aid our understanding of immune responses to tumours and assess novel targets with the aim of improving cancer immunotherapies.

What outputs do you think you will see at the end of this project?

Through studying the response of T lymphocytes and other white blood cell (immune cell) populations to cancer, our studies will enable us to:

1. Define the impact of cancers cells and their products on white blood cell function and metabolism

2. Determine how the availability of nutrients within the body affects the outcome of anti-tumour immune responses and tumour growth

3. Screen and target molecules that regulate immune activation in cancer

4. Test combinations of therapies to enhance clearance of tumours by the immune system

An immune response is frequently provoked during the early stages of cancer, yet one of the hallmarks of advanced cancer is the ability of tumours cells to evade these responses. Current immunotherapies seek to overcome the failure of immune responses and can broadly be placed in two main categories:

1) Drugs that enhance the responses and / or relieve suppression of immune cells already present within cancer patients.

2) Tumour-targeting immune cell populations that may be genetically modified to enhance their anti- cancer function, before injection into patients

Both pre-existing and transferred immune cell populations must overcome a characteristically hostile environment that is created within tumour masses. Our project will seek to gain an understanding of the responses of immune cells within these challenging environments, with a view to developing new approaches to improve immune function in cancer. Importantly, we suggest that this work will have implications for the development and future success of novel immunotherapies.

Tlymphocytes and other immune cells need to maintain a supply of nutrients such as glucose and amino acids to provide energy to sustain their responses in cancer. Evidence suggests that fast- growing tumour cells can out-compete immune cells for these vital

nutrient resources within tumours. Furthermore, inhibitory products present within tumours impact upon immune cell metabolism. How these conditions vary between different types of cancer / different locations within the body, and the role of individual nutrients in determining immune responses is not fully understood. Through our previous published work, we identified one particular nutrient as being important for T lymphocyte responses. In this project, we will define the role of nutrient availability in anti-cancer immune responses and test how manipulating T lymphocytes might improve their capacity to thrive in nutrient- deprived conditions. Furthermore, Identification of factors within tumours that impede immune cell metabolism may enable the development of ways to improve immunotherapies.

The ability of T lymphocytes to respond to cancer cells depends upon triggering through a cell-surface molecule termed the T cell receptor (TCR). Several current immunotherapies seek to improve the ability of T cells to respond to TCR triggering, whilst our previous published work has defined key mechanisms that regulate these processes. In the current project, we will seek to build on this previous work and determine the impact of deleting inhibitory factors within T lymphocytes with a view to improving anti-cancer immune responses. If successful, these approaches could be incorporated into the design of novel immunotherapies in the future.

The overall benefit of this work will be to increase our understanding of the regulation of immunity, identifying how and why immune responses fail and testing new approaches to revive anti-cancer responses. Therefore, this work has the potential for publication in cross-disciplinary scientific and medical journals. We envisage that this will also lead to new productive collaborations with clinical colleagues and industrial partners to enable us to translate our findings into real-world benefits.

Who or what will benefit from these outputs, and how?

A major benefit of this work will be to increase our knowledge of the mechanisms that regulate the induction and suppression of immune responses to cancer. Importantly, this knowledge will inform our future scientific aims and, through the refinement of our experimental protocols, will enable us to maintain the highest welfare standards for our experimental animal colonies.

Cancer represents a considerable health burden in society. A major goal of the proposed program of work is to test the role of pathways, nutrients and genes that are implicated in protective immunity to tumours. For example, using GA mice, we can test whether modification of target genes alters the ability of immune cells to respond to cancer and provide proof-of-principle for future studies to improve immunotherapies.

Important and immediate outputs for the work will include scientific publications and talks at national and international scientific meetings. The work will be of value and of great interest to fellow researchers in the fields of immunology, immunometabolism and cancer immunotherapy. The project will also help identify novel approaches to manipulate immune

cell responses in disease. Thus, it is expected that the knowledge gained from our animal experiments will be translated to pre-clinical human studies with our colleagues within our establishment's hospital campus, that could generate intellectual property rights and inform the generation of new human therapeutics in the future.

How will you look to maximise the outputs of this work?

Our research involves collaboration with groups within our institute as well as national and international colleagues and the current project will seek to maximise the benefit to all partners through:

- transfer of knowledge and expertise. For example, international collaborators will provide access to protocols and reagents for investigating metabolic processes whilst our group will provide in vivo samples

- provision of training and new skillsets to postgraduate student and postdoctoral researchers, thereby facilitating and enhancing their career development

- enhanced quality of scientific publications through complementary experimental approaches and technologies. In this regard, our in vivo approaches will complement studies using clinical samples and / or lab-based molecular techniques.

We will publish the results of our studies in leading scientific journals and present the work at relevant national and international conferences. We have previously accelerated the dissemination of our research by posting our results as "pre-print" publications to the bioRxiv server (bioRxiv.org). This enables us to share our findings months before acceptance in peer-reviewed journals as well as to receive feedback from the scientific community. We will continue to use social-media platforms (e.g. Twitter) as well as traditional media outlets to inform and engage with both the scientific community and non-specialist public on our own and related scientific research.

Species and numbers of animals expected to be used

- Mice: We anticipate using up to 4750 over the 5 years that this project will run. Note that that up to 500 mice will be transferred from protocol 1, under continuous use, to protocols 2 and 3.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The species of choice is the mouse as it can be genetically modified in a manner that is not currently possible with other mammalian species. Furthermore, the use of well-characterized inbred mouse strains maximises the reproducibility of in vivo research.

Importantly, a wealth of commercial reagents are available for the study of the mouse immune system and for intervention in and treatment of mouse models of cancer. We will investigate immune responses of young adult mice to ensure the reproducibility and cost-effectiveness of our studies.

Typically, what will be done to an animal used in your project?

The purpose of this project is to study immune responses to cancer. Therefore, in typical experiments mice will be injected with cancer cells either into the skin, a vein or the peritoneal cavity with or without substances or immune cells that we hypothesize will modulate the immune response and growth of tumours. We will study the growth of tumours in mice using advanced imaging techniques and may take blood samples to monitor circulating immune cells or nutrient levels. Experiments will vary in duration depending on the growth rate of tumours. This can vary substantially in different models from 7-70days. Wherever possible we will use techniques that have the least possible impact on animal welfare and undertake experiments of the shortest possible duration.

What are the expected impacts and/or adverse effects for the animals during your project?

Injection of cells or substances with a needle causes mild and transient pain at the injection site. In some circumstances we may use temporary anaesthesia for a few minutes to minimise discomfort for animals resulting from injections or for imaging procedures. Injection of cancer cells leads to the formation of tumours that in a minority of cases (<20% in protocols 2 and 3) may cause moderate suffering such as difficulty with breathing (lung tumours resulting from intravenous injection) or the accumulation of fluid in the peritoneal cavity. Such suffering will be short in duration as we undertake regular monitoring of mice and humanely cull any animals to prevent unnecessary suffering. Through the experiments, we hope to define fundamental aspects of the processes underpinning immune responses in cancer and assist in developing novel medicines to improve the treatment of these diseases.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of mice will experience mild or subthreshold suffering. For example, much of the analysis of experimental mice is initially carried out in the laboratory using fresh cells and organs from the mice generated by breeding. Therefore, ~80% of our mouse colonies will be humanely culled with no regulated procedures performed beyond being bred. For mice that receive tumour cell injections, <20% of mice will experience moderate suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our aim is to understand complex biological processes in immune cells, as this information will be most relevant to the design of future therapeutic approaches. Whilst some basic questions can be addressed using immortalized cell lines, such approaches have numerous limitations, most importantly that these cells are by definition quite distinct from primary cells. Immortalized cell lines frequently have mutations in key signalling and metabolic proteins which enable their survival and growth out with the body. As such, these genetic alterations render the analysis of signalling and metabolism in cell lines as quite distinct.

Wherever possible, we study immune cell function in the laboratory; e.g. tissue co-culture of lymphocyte populations with tumour cells. However, to understand the complex interplay between immune cells, cancer cells and environmental factors such as nutrients, requires the use of in vivo animal models. This is particularly prescient when the aim of the programme is to study the role that individual cell types and molecules play in these highly complex immune responses, as proposed here. These diseases involve the interplay of multiple tissues and factors that cannot be modelled using currently available tissue culture systems.

Which non-animal alternatives did you consider for use in this project?

We will study immune cells from healthy blood donor and cancer patient samples to complement our animal studies.

Why were they not suitable?

Study of human cells in the laboratory will enable us to begin to assess the translational implications of our animal work. Nonetheless, if we are to define mechanisms of immune regulation and metabolism in cancer and assess whether molecules can be targeted therapeutically, the use of some animal experiments is essential.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Depending on the consistency of the results obtained in an experiment, more or fewer animals are required to support or reject our hypotheses. We can use online mathematical tools or get advice from statisticians in order to perform statistical tests to assess the robustness and reproducibility of our experimental findings. We have used the results of our previous projects and data from similar experimental systems to predict how variable our experimental data are likely to be and therefore estimate how many animals will be required. In all cases, we use the minimum number required to generate meaningful data.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have substantial experience in using and setting up the experimental protocols detailed in this application. In this regard, in many cases estimates of animal numbers have already been established either through analysis of previous experiments or small-scale pilot studies to establish variability.

We also take advantage of online experimental design tools, particularly the Experimental Design Assistant from the NC3Rs. This tool enables researchers to input the key components from each stage of an experiment; from numbers of animals and numbers of experimental groups through to data measurement protocols and statistical analysis. This provides assistance in the design of robust and reproducible experiments that minimise the possibility for subjective bias in the measurement and analysis of data and reduce animal numbers to the minimum required to achieve the scientific objectives.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In all cases, we ensure that mice are bred in the most efficient manner. Where practical, established genetically-altered mouse breeding colonies are bred in a manner that all offspring will have the required genetic modifications and can be used for experimental purposes. The size, frequency, numbers and health of litters from breeding mice are closely monitored by animal facility staff and researchers so as to maintain efficient colonies of adequate but not excessive animal numbers.

In many cases, we can minimise animal numbers by using multiple tissues from one animal to assess several experimental parameters; e.g. tumour tissues, blood and lymphoid organs such as the spleen can all be harvested and assessed in the laboratory.

When setting up new experimental protocols, pilot studies using small numbers of animals will be performed to enable us to determine the optimal strategy required to answer the experimental objectives.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For protocol 1, the majority of mouse lines have no defects beyond alterations in or loss of immune cell populations and suffer no ill effects when they are maintained in filtered cages in our specific-pathogen free animal facility. In addition, we will use mouse models of cancer to model immune responses and therapeutic approaches to the treatment of cancer (protocols 2 and 3). In these experiments, cancer cells grown in the laboratory are injected into animals with a needle and a tumour will form at the injection site or in the case of intravenous injection, in the lungs. It is therefore unavoidable that some animals will experience suffering. The severity of these approaches will be kept to a minimum with

~80% of mice in protocols 2 and 3 experiencing a mild form of suffering. For some mice, growth of tumours may result in moderate levels of suffering. However, regular monitoring of animal symptoms as well as measurement of tumour growth by imaging techniques and/or visual inspection ensures that mice do not experience unnecessary suffering and are humanely culled. Through this work, we will be able to address fundamental questions about the immune response to cancer and help in the development of improved medicines to treat these diseases.

Why can't you use animals that are less sentient?

We wish to model cancer and immune responses in adults. Importantly, studies of adult mice are widely recognised to be relevant for the study of immune processes and cancer whilst many therapeutic breakthroughs have been made following initial mouse studies e.g. the use of several immunotherapies for the treatment of cancer. Furthermore, the ability to genetically manipulate mice is essential to test our specific hypotheses whilst the availability of key reagents and therapies to assess mouse immune function is central to our experimental design. With this in mind and to best of our knowledge, there are currently no non-sentient or lower sentient alternatives to the use of adult mice for our work.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals undergoing experimental protocols are monitored routinely by researchers and animal facility staff using specific checklists to assess wellbeing, discomfort and suffering. The frequency of monitoring is increased upon the development of specific symptoms. Anaesthetic is used whenever appropriate to minimise pain/discomfort and advice from

veterinary surgeons sought whenever unexpected or unexplained signs of distress, discomfort or pain develop.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All animal suffering will be limited to unavoidable procedures required for the performance of our experimental protocols. For general guidance on the conduct and refinement of animal experiments, the NC3Rs provide a wealth of resources and information online (https://nc3rs.org.uk/refinement- refining-animal-experiments-minimise-pain-suffering-and-distress). Relevant academic literature on the care and use of mice in research will be referred to, including NC3Rs published guidelines (1).

Furthermore, specific guidelines on the conduct of cancer research in experimental animals will be adhered to at all times (2).

1. Prescott MJ, Lidster K (2017). Improving quality of science through better animal welfare: the NC3Rs strategy. Laboratory Animal 46:152-156.

2. Workman P et al (2010). Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer 102:1555-1577.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will continue to subscribe to regular e-mails from NC3Rs, that contains updates on best practice in animal husbandry and experimental design. All researchers undertaking experimental protocols under this project will review relevant scientific literature, particularly in specialized peer-reviewed journals such as Laboratory Animal, that pertain to the conduct and refinement of mouse cancer models and will implement any advances in the design of subsequent experiments.

166. In vivo pharmacokinetic and pharmacodynamic properties of potential new treatments for cns and other disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Drug discovery, Pharmacokinetics, Pharmacodynamics, CNS penetration

Animal types	Life stages
Mice	neonate, juvenile, adult
Rats	neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To evaluate the properties of compounds that are being developed as potential drugs for the treatment of central nervous system (CNS) and other disorders.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There remains a large, unmet medical need for the treatment of a variety of central nervous system (CNS) and other disorders. This is exemplified by age-related diseases, such as Alzheimer's disease, that are increasing in prevalence as the population ages. However, there is an increasing awareness of the devasting personal and societal impact of psychiatric disorders and mental ill-health. The collective impact of all these disorders is a testament to the inadequacy of existing therapies and the need for new and innovative treatments.

As our understanding of the mechanisms underlying a variety of diseases increases, then so our ability to identify novel potential strategies for treating those diseases also improves. More specifically, we can identify particular proteins involved in the disease process and as part of the drug discovery process we can identify synthetic molecules that alter the function of those proteins in the test-tube and in in vitro cellular models. However, despite the technological advances in computer (in silico) and in vitro (test-tube and cellular assay) methodologies, there nevertheless remains a critically important need to evaluate what effect a drug has on the intact, live animal (pharmacodynamics) as well as what effect the animal has on the drug (pharmacokinetics). It is the pharmacodynamics and pharmacokinetics of novel compounds that are the focus of this Project Licence.

What outputs do you think you will see at the end of this project?

Over the five-year period of the project, the primary outcomes will be the identification of novel drugs for the treatment of CNS and other (e.g., cancer, immunological) disorders. As regards CNS disorders, the host institution is currently working on early-stage drug targets for Alzheimer’s disease, Huntington’s disease, anxiety disorders, schizophrenia, bipolar disorder and treatment-resistant depression and therefore patients suffering from any of these disorders could be potential beneficiaries from the studies covered by this licence. In addition, drug discovery projects in non-neuroscience-related therapeutic areas will be established as and when the scientific opportunity arises.

The primary outputs of the work conducted under this licence will be novel small molecules, the most advanced of which will become drugs for human use. Compounds that do not meet all the criteria required for a drug suitable for human use, will be made available to the wider scientific community to enable mechanistic studies into the protein and/or disease of interest.

Who or what will benefit from these outputs, and how?

The primary outputs from the work conducted under this licence will be new drugs for the treatment of CNS and/or other disorders. Within the 5-year period covered by this Project Licence we would anticipate that drugs for certain of our projects will progress into early-stage (Phase 1) clinical studies and in the longer term (5-10 years following commencement of this licence) we would hope that at least one drug will have achieved Regulatory Approval and is being marketed for either a CNS or other disease.

How will you look to maximise the outputs of this work?

The work conducted under this licence will contribute to the Intellectual Property that describes the compounds/potential drugs of interest. This Intellectual Property will be protected by Patent Filings which are critical to the licensing of our drugs to the pharmaceutical companies that have the required clinical trial expertise needed to achieve the Regulatory Approval (by the FDA, EMA or MRHA) needed to licence the drugs and make them available to the patient population.

During the drug discovery process, we will identify pharmacological tools compounds that are suitable for further preclinical characterisation but, for whatever reason, cannot be progressed into clinical studies. These will be made available to the wider scientific community and will be "advertised" by means of standard academic procedures (meeting attendance, poster presentations etc).

Species and numbers of animals expected to be used

- Mice: 5250
- Rats: 9500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will typically use young adult mice or rats since these are standard species for examining the effects of drugs on animals (pharmacodynamics) and the effects of the animal on the drug (pharmacokinetics). Previous studies have highlighted the value of these animal studies in predicting the behaviour of drugs in man.

Typically, what will be done to an animal used in your project?

A typical experiment will involve the injection of compound into the animal followed by the measurement of some parameter related to the effect of the drug (pharmacodynamics) or how long the drug lasts in the animal (pharmacokinetics). Such parameters might include the measurement of drug concentrations in the blood plasma and/or brain or the effects on brain function (e.g., protein expression or behavioural pharmacology). The majority of experiments (>90%) will involve the administration of a single dose of test compound followed by termination and sample collection (e.g., the brain) within a day of dosing the compound.

What are the expected impacts and/or adverse effects for the animals during your project?

The anticipated adverse effects include mild/moderate effects either as a result of the dosing procedure itself (e.g., transient immobility) or, in <1% of animals, drug-related adverse pharmacological effects.

More specifically, we are generally identifying and developing drugs that affect the balance of nerve cell excitability and nerve cell inhibition. This “Ying and Yang” of brain function requires that we subtlety modulate nerve cell function (i.e., increase or decrease nerve cell excitability or inhibition) without producing side-effects that characterise the extremes of nerve cell excitability (i.e., seizures) or inhibition (i.e., sedation or unconsciousness). These risks are considerably mitigated by our characterisation of the in vitro pharmacology of compounds, but there nevertheless remains a small risk that some of these side-effects may manifest themselves during in vivo testing. Compounds which produce significant Adverse Events will not be of interest for additional in vivo profiling since such liabilities will clearly prevent further preclinical development of such compounds. Hence, any observed Adverse Events will be sufficient to make a No-Go decision for the further in vivo characterization of such a compound.

It is important to emphasise that novel compounds due to be tested in live animals will be been characterised in a variety of laboratory tests. These laboratory tests are critical in order to prioritise compounds for live animal tests and to exclude compounds that will be of only limited interest and value to the overall drug discovery project.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

For both mice and rats, the overall severity of effects experienced by animals is anticipated to be minimal with the vast majority (>95%) showing no drug-related adverse effects. Of the remainder, most are anticipated to show mild/moderate effects either as a result of the dosing procedure itself (e.g., transient immobility) or possibly a longer term, drug-related immobility (e.g. sedation).

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The drug discovery process involves the synthesis of multiple (several hundred) novel compounds in which iterative changes are made to a molecule in order to improve its

properties. Each compound needs to be tested in order to ensure that it retains the desired properties and while most of these tests are conducted in test tubes or in cell cultures (in vitro) at some point, a few, more advanced compounds need to be tested in live animals (in vivo). Importantly, novel compounds will not be directly tested live animals without prior in vitro characterisation.

Depending upon the specific project and the scientific question, partial replacement may be possible in so far as cells or tissues may be used from animals that have not undergone any procedures other than killing by a humane method.

There are other key aspects of the drug discovery process that based upon our current understanding of the intact, living organism cannot be replaced by other methods in vitro or computer prediction (in silico) methods. Such aspects include, for example, how rapidly an animal will metabolise a novel compound, to what extent a compound can cross the blood-brain barrier and penetrate into the CNS and engage with the target of interest (e.g., receptor occupancy, enzyme inhibition), and what the functional consequences are of a drug modulating the function of its target protein.

Which non-animal alternatives did you consider for use in this project?

There are currently no non-animal alternatives for certain aspects of the drug discovery process. However, wherever possible we will use in vitro surrogate assays to deprioritise compounds prior to the in vivo stage. Such assays can include in vitro assays of drug metabolism (liver microsome and/or hepatocyte assays), blood-brain barrier permeability and efflux (drug transport across Caco-2 cell monolayers). Moreover, in silico predictions of metabolic "soft-spots" on compounds of interest will also be used to predict - and therefore deprioritise - compounds that are likely to be inherently unstable in vivo.

Why were they not suitable?

The complexities of the systems involved, for instance the brain, mean it is not possible to predict, either in silico or via in vitro cellular or other assays, what the response of the live animal will be. This is particularly the case for the brain where the intricacies of the nerve cell connections and their circuitry mean it is not possible to predict, for example, what the functional consequences of a compound penetrating across the blood brain barrier and engaging the target protein of interest will be.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

This estimate is based upon the usage under the previous 2016-21 Project Licence (5250 mice and 9500 rats) which supports 3 projects. That project supported three main projects, supported by four members of staff and this current project proposal will support those existing projects plus an additional three new projects. However, not only are these new projects at an earlier stage that requires less animal usage, but the existing projects are maturing such that their animal usage will also be reduced as they move closer to or into the clinical testing phase. Collectively, these projects are funded by roughly £10m of research funding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The data from the in vivo experiments described in this Project Licence form part of a collective data set that is used to prioritise compounds for further progression (e.g., from target engagement studies to functional - behavioural pharmacology - studies). Since compound prioritisation is a combination of the combined in vitro, in vivo and other "druggability" data sets, it is often sufficient to generate qualitative data to differentiate compounds that, for instance, give penetration into the CNS that is good, bad or in-between. Such qualitative data can be generated using smaller group sizes (e.g., n=3-5) compared to quantitatively demonstrating that Compound X gives significantly greater CNS penetration than Compound Y.

For studies in which quantitative comparisons of the functional effects of compounds are required (e.g., the effect in drug- versus vehicle-treated animals), experiments will be designed in accordance with the NC3R's Experimental Design Assistant (https://eda.nc3rs.org.uk/) and follow guidance from the MRC (https://mrc.ukri.org/funding/guidance-for-applicants/4-proposals-involving-animal-use/).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our in vivo studies will be guided by the principles of the ARRIVE guidelines (https://arriveguidelines.org/) and where necessary, pilot studies will be conducted according to NC3R guidelines (https://nc3rs.org.uk/conducting-pilot-study).

For studies in which samples need to be collected for assay development work, we will endeavour to use tissues obtained from animals no longer required for their primary purpose. For in vivo assay development, relatively small pilot studies will be conducted to: a) assess feasibility; and b) conduct power analyses should they be required for quantitative analyses (e.g., does Compound X significantly alter parameter Y in relation to vehicle-treated animals?).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This Licence will generally use young adult mice or rats. The basic experimental design is to dose animals with test compound and measure the pharmacodynamic effects that compound has on the animal (for instance effects on a biomarker of target engagement or a functional response, such as behaviour) and the pharmacokinetic effects the animal has on the test compound in terms of absorption, distribution, metabolism and excretion (e.g., penetration in the CNS across the blood-brain barrier).

Animals will normally receive a single dose of test compound and will be killed a short time (<24 h) later, thereby minimising any potential lasting harm.

Assays will generally be selected on the basis of pharmaceutical industry standards. For example, we will use behavioural assays that are well recognised to provide the best construct and/or face and/or predictive validity in relation to the phenotype they are modelling.

Why can’t you use animals that are less sentient?

Generally, young adults will be used rather than more immature animals since they normally more accurately model the physiological aspects we are trying to assess. For example, younger animals do not possess a mature blood-brain barrier and therefore cannot be used to measure the ability of a compound to cross the blood-brain barrier. On occasion, it may be necessary to use non-adult (e.g., new born or juvenile) animals if, for instance, the protein of interest is developmentally regulated such that expression is much higher in new-born or juvenile animals compared to adults.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our species of choice will be the rat since any data that are generated as part of this project are then consistent with subsequent safety and toxicity studies that are required by the regulatory bodies to be conducted in rat. However, where necessary, we will use mice to generate data that are consistent with other studies showing the effects (efficacy) or side-effects of drugs in mice (e.g., efficacy studies in particular strains of transgenic mice to be covered outside of this Project Licence).

As regards animal welfare, where necessary, pre-meetings between researchers and animal care staff will take place to evaluate and put in place monitoring systems to identify potential welfare issues arising from specific protocols. Environmental enriched housing is provided to all of the animals throughout these procedures.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our general approach to our choice of the most appropriate animal model will be informed by resources such as 'Systematic reviews of animal studies' webinar (https://vimeo.com/421550517/65395c0b20) and the NC3Rs Systematic Review Facility (SyRF; https://www.nc3rs.org.uk/camaradesnc3rs- systematic-review-facility-syrf). Moreover, we will seek input from our colleagues within the pharmaceutical industry to get their advice on the relative decision-making values of the various alternatives for in vivo assays.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Subscription to NC3Rs newsletter.

167. Brain networks in epilepsy and neurodevelopmental disorders

Project duration

5 years 0 months

Project purpose

Basic research
Translational or applied research with one of the following aims:
- Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
- Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

Epilepsy, Anatomical Networks in the Brain, Genetic Models, Neurodevelopmental Disorders, New Therapies for Epilepsy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, aged
Rats	neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to better understand the anatomical pathways that allow seizures to spread in the brain. This will allow us to better identify the causes of epilepsy and develop improved ways to treat the disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Anti-epileptic drugs and surgery to remove epileptic tissue are ineffective in controlling seizures in one third of patients with epilepsy. A major obstacle that prevents us from developing better therapies is that our knowledge of how seizures in epilepsy spread through the brain is limited. Seizures that spread throughout the brain are also particularly dangerous and can lead to people dying from a seizure, if critical areas of the nervous system that control the heart or respiration stop functioning are affected. Furthermore, there are new technologies being tested to treat different diseases including drugs with less adverse effects, electrical stimulation of specific brain areas and, genetic therapies to change the activity of individual populations of brain cells. However, in order to target these effectively to reduce seizures spreading dangerously through the brain, it is necessary to understand how seizures do so. Once we know this, we can change the activity of a specific area and stop seizures.

This is similar to a network of pipes in a house, if there is a leak that allows damage to occur then we need to figure out where that leak is. Once we do, we can direct therapies to block this leak and prevent the damage associated with the disease.

What outputs do you think you will see at the end of this project?

New treatments are urgently needed for epilepsy. The ultimate goal of this project licence is to understand how seizures spread through the brain and stop those seizures from spreading by modulating the activity of a specific groups of cells. The outputs of this work will be scientific articles that highlight this and motivate clinicians to undertake interventions in patients developed in animal models.

We will also identify biomarkers of the disease that can be tested in clinical patients and can be used to diagnose patients and quantify improvement in therapies.

Who or what will benefit from these outputs, and how?

The outputs will eventually benefit patients and their families. Epilepsy can affect people of any age but children under 5 and adults over 60 have a higher likelihood of developing chronic seizures. 1 in 103 people in the UK suffer from epilepsy and a third of those patients is not able to attain control of their seizures with anti-epileptic drugs and must undergo surgery to remove epileptic tissue. In the UK 1150 people died of epilepsy related causes in 2009. The cost of epilepsy is £244 million per year. A prominent clinician has noted that “…epilepsy has an impact on the education of children and young people". One third of children with epilepsy have continuing seizures, which are likely to affect their concentration, behaviour and/or attendance levels at school. Even for the two thirds of children whose seizures are controlled, the anti-epileptic drugs they are taking can impact upon their concentration and learning. Unemployment rates are doubled for people with

epilepsy compared to those without a disability. From 2005 to 2011 the number of people suffering from epilepsy has risen by 32%. Thus, epilepsy has a major impact on the UK's economy and the prosperity of its population.

The outputs will also help other epilepsy researchers as well as clinicians in better understanding the disease.

How will you look to maximise the outputs of this work?

The results from this work will be shared at international conferences for basic scientists and neurologists. We will also engage in public engagement through donor groups, patients, charities and social media.

Species and numbers of animals expected to be used

- Mice: 8000
- Rats: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are studying complex brain anatomy that leads to seizures spreading through the brain. This type of research requires nervous systems that are similar in structure to that in humans to increase our understanding of the disease and develop new therapies. Mice and rats have brains that are similar in structure to humans. We study them from early life to late adulthood as different types of epilepsy occur at different points in life. In human genetic epilepsies and neurodevelopmental disorders, such as severe autism or intellectual disabilities, seizures can block or delay normal brain development.

Therefore, we study young animals to mimic the human paediatric cases. While other types of epilepsy occur in adulthood or in later life.

Typically, what will be done to an animal used in your project?

Animals will be made epileptic, where seizures emerge, either by genetic manipulation or through stimulation of the brain. Animals will be implanted with electrodes in different locations in the brain that we know are interconnected with each other. We will study how seizures move through these different areas by recording from these animals. Animals will be recorded for multiple weeks 24 hours per day. This is done through wireless transmitters that prevent animals from being connected and allows them to move freely in their enclosures. We will also perform interventions to block seizures by stimulating specific brain cells in order to develop new potential therapies.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will develop epilepsy therefore animals will have seizures. These are usually of the kind where animals will lose consciousness, as human patients that have these type of seizures do not remember the seizure event.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

95% of animals will be of moderate severity, with 5% being mild (both in mice and rats).

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Animals are needed as we do not know enough of how seizures move through complex brains that share a high level of similarity with humans. This is only possible in animals.

Which non-animal alternatives did you consider for use in this project?

I have considered computer models, human patients and in vitro models of epilepsy.

Why were they not suitable?

Our knowledge of brain networks during seizures is still not sufficient to be able to make these models informative. I hope to be able to gain knowledge in this field through these experiments that may be used to generate these models in the future. These experiments cannot be tested in humans as we are looking to understand how epilepsy comes about in the brain and to develop treatments, however this will take time. It is also not possible to use brain extracts in our experiments as these do not mimic the human version of seizures accurately.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I have utilized statistical analyses to come up with estimates of how many animals are needed per experiment. These analyses are based on previous experience with this type of experiment and expectations of an expanding number of researchers and projects over the next five years.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will utilize pilot studies to ensure that there is a high likelihood of an experiment that is capable of testing a proposed hypothesis. Furthermore, different data types will be collected from each animal including electrical, video and anatomy, which will generate information about different aspects of the disease.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Most animals will generate hundreds of hours of brain activity. These large data sets will be analysed in great detail utilizing advanced analyses such as machine learning to develop seizure prediction, biomarkers for disease to allow for better diagnosis and new potential avenues of research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use animal models of induced epilepsy through chemical or electrical stimulation as well as genetic models where specific mutations that predispose patients to epilepsy and neurodevelopmental disorders have been generated in mice and rats.

Why can’t you use animals that are less sentient?

In order to study how seizures propagate through complex brain networks that are clinically relevant it is necessary to use brains that highly approximate human brain.

Rodents have the overall same structure as human brain with a cortical mantel and underlying structures. This cannot be done in less sentient animals that do not have brain structures that allow for higher cognition. Furthermore, epilepsy often affects elements of cognition and it is important to understand how cognition is affected in our models and how these comorbidities are affected by how seizures spread through the brain.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are primarily using wireless technologies to perform long-term recordings in these animals. This allows us to study epilepsy more accurately as seizures in patients occur spontaneously, so we need 24 hour long term recordings. However, since the recordings are wireless animals are free to move in their cage and often can be housed in groups, which lowers stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

"Opportunities for improving animal welfare in rodent models of epilepsy and seizures" J. Neurosci. Methods

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I will consult scientific literature on 3Rs, take heed of local announcements of the 3Rs in my institution and follow 3R accounts on social media.

168. Towards mechanistic understanding and improved treatment of nervous disorders

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

neurological disorders, psychiatric disorders, genes, brain, behaviour

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To enhance understanding of how genetic alterations pertaining to human nervous disorders affect brain structure/function and behaviour.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

A wide variety of conditions come under the umbrella term 'nervous disorders' because they impair the normal functioning of the brain and affect behaviour. Neurodevelopmental disorders, such as autism and learning disability, affect the development and structure of

the brain. Epilepsy is characterised by unpredictable epileptic fits or seizures. Psychiatric disorders, such as schizophrenia, alter behaviour, perceptions and mood. Neurodegenerative disorders, such as Alzheimer's disease, are characterised by the death of nerve cells in the brain. Although these disorders have different characteristics, the first sign that something is wrong is often a change in behaviour. For example, an infant with learning disability may not begin saying simple words at the expected age, or an elderly person with the first signs of Alzheimer's disease may get lost walking to the shops.

Nervous disorders can be difficult to treat effectively with currently available medications, which at best provide only symptomatic relief - not tackling the underlying cause of the disorder. For some patients, symptoms may be alleviated by the prescribed treatment, but the disease may still gradually get worse (known as disease progression). Treatments often have unwanted side effects (e.g. tiredness, tremors), which themselves may affect patient well-being, causing some patients to stop taking their medication - which could lead to symptoms returning. For other patients, symptoms cannot be alleviated at all with current treatments. There is thus an unmet need for improved treatments for nervous disorders.

It is important to undertake work to achieve the aim of this project because patients with nervous disorders often have changes in their genes compared with healthy individuals. Through the use of DNA sequencing, scientists are able to identify genetic changes that are likely to contribute to the patient's condition. In this way, we are beginning to gain an understanding of what is causing the brain to function abnormally. For example, my research group recently identified an altered gene as the likely cause of a form of learning disability. Better knowledge of what causes the onset of each nervous disorder, and the possible worsening of symptoms, will help scientists to develop better treatments.

Given the strict limitations upon invasive approaches in clinical studies, it is difficult to do the necessary experiments in human patients. Unlike taking a blood sample or a skin biopsy, it is hazardous to a patient's health to take a biopsy from their brain. Therefore, we need to consider alternative ways to study how disease-associated genetic changes cause the brain to function abnormally. One way is to introduce the same genetic change found in human patients into cells grown in the lab. This cell culture approach may tell us how the genetic change affects aspects of the health of cells grown in a Petri dish, but it cannot tell us how the genetic change affects a whole brain made up of millions of cells, nor how it affects behaviour. A more informative approach is to introduce the same genetic change into mice, and study its effects on their brain and behaviour. This is a valid approach because mice and humans share all but ~1% of each other’s genes, the brains of all mammals have the same basic components and

structure, and mice exhibit a range of behaviours that can be measured and are similar to those exhibited by human beings (e.g. learning and memory, anxiety, social interactions).

What outputs do you think you will see at the end of this project?

Outputs in the form of published scientific articles and presentations to scientific conferences and patients' groups at the end of this project are expected to emerge from new information gained on how genetic alterations pertaining to human nervous disorders affect brain structure/function and behaviour.

Who or what will benefit from these outputs, and how?

In the short-term, these outputs would benefit affected families through genetic counselling and carrier screening, and facilitate the genetic diagnosis of other patients. The latter would allow affected families with the same genetic diagnosis to contact each other and provide mutual support, and allow doctors to prescribe (and patients to receive) existing treatments known to be beneficial in other patients with the same genetic diagnosis. Other beneficiaries in the short-term would be scientists trying to understand the molecular mechanisms of nervous disorders, and those trying to develop better treatments. It may also benefit scientists studying the normal biological functions and development of the brain, because a useful way of understanding how something works is to find out what happens when it goes wrong. In the longer term, patients and their carers would be obvious beneficiaries of the development and availability of better treatments.

How will you look to maximise the outputs of this work?

I would look to maximise the outputs of this work in multiple ways.

1. By collecting and archiving tissue from the brain and other organs of genetically altered mice and wild-type littermates, so that collaborators and ourselves can continue to study the effects of genetic changes after the mice have died.

2. By collaborating with other scientists who use techniques (under separate authority) in which my research group has little expertise.

3. By video recording mouse behavioural tests so that they can be analysed offline to look for subtle effects not detected initially.

4. By using non-animal approaches, such as differentiated neurones, to corroborate research findings.

5. By publishing successful approaches in high-impact journals, but also attempting to publish less successful approaches.

6. By disseminating research findings to patients' group and clinicians.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

For several reasons, the project will use mice.

(i) There is extensive knowledge of their neuroanatomy, physiology, and genome.

(ii) They have a similar genetic make up to humans, sharing all but ~1% of each other's genes.

(iii) The ability to manipulate the mouse genome permits the introduction of genetic alterations pertaining to human nervous disorders.

(iv) The techniques we intend to use have been designed for mice and shown to be successful, including a variety of behavioural tests that allow us to assess nervous disorder-related behaviours.

Given the strict limitations on studies with human patients to find out how genetic changes affect their brain structure/function and behaviour, we will use genetically altered mice that harbour genetic changes shared with nervous disorder patients. There is growing recognition that manifestations of many human disorders most likely emerge from an underlying developmental and cellular biology shared in large part with other species.

When we are using genetically altered mice to study neurodevelopmental disorders that begin early in life, such as autism, we will observe juveniles and conduct experiments on young adults. When we are using genetically altered mice to study neurodegenerative disorders that begin later in life, such as Alzheimer's disease, we will observe young adults and conduct experiments on mature adults and aged mice to determine how the disease progresses with age.

Typically, what will be done to an animal used in your project?

If only one copy of a genetic change is required to cause the human nervous disorder under study, we will breed mice harbouring one copy of the genetic change (known as heterozygous) with mice harbouring two copies of a normal gene (known as wild-type); we would expect 50% of the offspring to be heterozygous and 50% to be wild-type. If two copies of a genetic change are required to cause the human nervous disorder, we will breed heterozygous males to heterozygous females, and would expect 50% of the offspring to be heterozygous, 25% to be wild-type, and 25% to have two copies of the genetic change (known as homozygous). Typically just after weaning at 3-4 weeks of age, each pup will have a notch of tissue clipped from its ear to identify it and for DNA analysis to determine its genotype, i.e. whether it is heterozygous, homozygous or wild-type.

Typically, adult mice will be habituated to handling for 7 days prior to behavioural testing comprising a battery of up to 8 tests, including no more than one test involving electric

shock, one test involving food restriction, and one test involving water restriction. The duration of an individual behavioural test can vary from 5 minutes to 2-3 weeks, if a test requires extensive training of the mice. The behavioural testing would usually be completed within 3 months. Occasionally, to assess disease progression, 4 of the 8 tests may be repeated no more than twice, after a period of at least 3 months, such that any one animal will be subject to a maximum of 16 behavioural tests in their lifetime.

Occasionally, a substance (e.g. an experimental drug treatment for the nervous disorder) may be injected into the mice prior to the commencement of behavioural testing. At the end of behavioural testing, the mice may be humanely killed (Schedule 1), and have their brain and organs collected, or may be retained for conventional breeding (Protocol 1 or 2) or 14C-2- deoxyglucose functional brain imaging (Protocol 7) if they have not been subject to administration of substances or surgery.

Occasionally, mice may be subject to a surgical procedure for a variety of reasons, including implantation of electrodes into the brain (to record electrophysiological signals during sleep and seizures); to facilitate collection of fluids from discrete parts of the brain; or to implant a mini-osmotic pump (for continuous infusion of drugs).

What are the expected impacts and/or adverse effects for the animals during your project?

Most of the genetically altered mouse lines bred for use in this project do not show any adverse effects (a mild rating). However, three of the genetically altered mouse lines show reduced weight gain compared with wild-type littermates (a moderate rating). In one of these lines, homozgous mice also exhibit unusual repetitive hindlimb jumping in the home cage. Heterozgous mice in another of these lines exhibit stress-induced seizures in the home cage (a moderate rating), which are occasionally fatal (a severe rating). When an adverse effect is caused by the genetic change that the animal harbours, it would typically be lifelong.

Most of behavioural tests used in the project do not have adverse effects (a moderate rating). However, one of the protocols includes administration of a convulsant substance to evaluate seizure susceptibility. At the lowest dose, the convulsant is not expected to induce seizures in wild-type mice, but it may induce seizures lasting a few minutes in mice with increased susceptibility to seizures (a moderate rating). At a 1.75 times higher dose, the convulsant is expected to induce seizures in wild- type mice (a moderate rating).

Three of the protocols include use of surgical procedures (a moderate rating). The following adverse effects are expected during surgery: corneal drying (occurs regularly under anaesthesia), respiratory depression (occurs routinely), decreased body temperature (occurs routinely), and pain (occurs routinely). However, these adverse effects will be ameliorated and controlled using appropriate measures (e.g. use of appropriate analgesia).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Mice
- Mild: 50%
- Moderate: 47%
- Severe: 3%

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Biochemical, anatomical, physiological, pathological and behavioural assays all contribute to understanding the functional consequences of a genetic change. Behavioural abnormalities, such as memory loss or convulsions, are the primary symptoms and diagnostic indicators of nervous disorders. Behaviour is an emergent property of brain function, involving coordinated activity both within and external to the CNS. Changes to genes and their expression may affect brain functioning, and hence behaviour, at multiple levels.

Unlike taking a blood sample or a skin biopsy, it is hazardous to a patient's health to take a biopsy of their brain. Given the strict limitations upon invasive approaches in clinical studies with human patients, we need to consider alternative ways to study how disease-associated genetic changes cause the brain to function abnormally.

Whilst in vitro model systems, such as cultured cells, have enhanced our knowledge of the cellular mechanisms through which genetic changes act, they have very limited utility for determining how genetic changes confer risk for nervous disorders, primarily because cells do not exhibit behaviours. As behaviour can only be studied in intact living animals, it is necessary to use animals to achieve the aim of this project.

Our approach is to introduce the disease-associated genetic change into the mouse genome, and find out how it affects the mouse's brain and behaviour. This approach is valid because humans and mice share all but ~1% of each other’s genes, the brains of all

mammals have the same basic components and structure, and mice exhibit a wide range of behaviours that can be measured and are similar to those exhibited by human beings (e.g. learning and memory, anxiety, social interactions).

Which non-animal alternatives did you consider for use in this project?

We recently began collaborating with a colleague to develop non-animal alternatives for the study of nervous disorders, namely 3-dimensional cultures called organoids. Genetic changes identified in patients with nervous disorders are introduced into human stem cells grown in the lab, which can then be turned into nerve cells and ultimately cultured into brain organoids (lab-grown 'mini-brains'). As human brain organoids are reported to recapitulate some features of the human brain, they could be used as alternatives to mice for studying the effects of genetic changes on brain development and structure.

Why were they not suitable?

Human brain organoid models are not currently suitable for use in this project because the technology is still at an early stage and too limited for organoids to replace animals. In future, in vitro investigations using organoids may provide some information on how genetic alterations pertaining to human nervous disorders affect brain structure/function, but not behaviour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimated number of mice used in the project is based on the following considerations.

- The number of genetically altered mouse lines to be used.
- The length of the project.
- The number of mice per experimental group (i.e. sample size) required to detect the effects of genotype (homozygous/heterozygous vs. wild-type) and sex.
- The number of mouse lines bred from heterozygous males and heterozygous females (i.e. intercrossing).
- The number of mouse lines bred from heterozygous males and wild-type females.

- The typical number of tests to which each mouse will be subject.
- Previous experience of running similar projects under two previous project licences.
- The likelihood of obtaining further grant funding.

These considerations have determined our overall plans for mouse breeding, maintenance and experimentation.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

General strategies that reduce the number of animals in each experimental group necessary to obtain statistically significant results will be adopted whenever appropriate. We will ensure reduction by writing a protocol for each experiment, which will include statistically designed sample sizes (by power calculations) and by searching the literature to ensure experiments are not unnecessarily duplicated.

Typically, sample sizes are estimated using statistical methods which result in group sizes of 12 being necessary to achieve satisfactory results. Such calculations have determined our overall plans for mouse breeding, maintenance and experimentation.

To avoid the necessity of breeding new cohorts for each behavioural test, each cohort will be subject to a battery of tests, rather than a single test. Battery testing has the advantage of reducing the number of animals required. Although this approach may lead to different results compared to naïve cohorts, large differences are unlikely. Hence, although this approach has limitations, the benefits outweigh the costs.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Breeding protocols will be designed to ensure that only the required number of animals are bred, to minimise wastage. The number of new mice born within each genetically altered line will be monitored on a monthly basis to ensure that the supply of mice for experiments or line maintenance is appropriate. If the supply of mice is excessive, the number of breeding animals will be reduced by Schedule 1 killing.

When studying a homozygous genetic change, mouse numbers could be reduced further by breeding independent cohorts of wild-type and homozygous mice for comparison. However, to minimise variation, we will instead intercross heterozygotes to generate sex-matched littermate controls for our experiments, which is standard practice for behavioural studies.

To avoid the necessity of maintaining animals solely for conventional breeding, animals that have been subject only to non-invasive behavioural tests, without administration of substances or surgery, may be maintained for conventional breeding.

To avoid an excess of post-weaning mice of unknown genotype on the shelf, we will strive to ear notch mice and identify genotypes as soon as possible after weaning. In this way, mice of a particular genotype and sex that are not required for experiments or breeding can be killed (Schedule 1) before reaching sexual maturity.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Medical conditions that come under the umbrella term 'nervous disorders' typically impair the normal functioning of the brain and affect behaviour. Behavioural abnormalities, such as memory loss or convulsions, are often the primary symptoms and diagnostic indicators of nervous disorders. Patients with nervous disorders often have changes in their genes compared with healthy individuals. Through the use of DNA sequencing, scientists are able to identify genetic changes that are likely to contribute to the patient's condition.

The ability to manipulate the mouse genome permits the generation of mice with the same genetic change as in human patients. The genetically altered mice are studied to determine how the genetic change affects their brain and behaviour. This is a valid approach to understanding more about human nervous disorders because mice and humans share all but ~1% of each other’s genes, the brains of all mammals have the same basic components and structure, and mice exhibit a wide range of behaviours that can be measured and are similar to those exhibited by human beings.

To measure nervous disorder-related behaviours in mice, they will be subject to a series of behavioural tests that are not expected to cause any lasting pain or distress. The techniques we intend to use have been designed for mice and shown to be successful. During the last project licence, it was realised that the active avoidance test, which includes administration of electric shocks over a 5-day period, could be adequately replaced by the less-stressful passive avoidance test, which we were already using.

Similarly, it was realised that the forced swim test, which is classified as severe, could be adequately replaced by a 'water exposure' test in which the mouse is placed in a container of water that is sufficiently shallow to permit the mouse to touch the floor with its hind paws, such that the mouse is not forced to swim.

If a genetically altered mouse line exhibits behaviours that replicate abnormalities exhibited by human patients, we may subsequently use it to test prophylactic or therapeutic strategies. Dosing with drugs by injection will cause only transient pain.

To quantify the degree to which genetic changes found in epilepsy and related nervous disorders affect susceptibility to seizures, mouse lines harbouring these genetic changes will be subject to procedures that may induce seizures. However, the seizure-inducing procedures that we have selected are designed to induce seizures only in mice with an increased susceptibility to seizures, not in wild-type control animals.

Why can’t you use animals that are less sentient?

Behaviour can only be studied in awake, living organisms, so animals that have been terminally anaesthetised would be unsuitable. Although epilepsy, fragile X syndrome and other nervous disorders can be studied in lower model organisms, most notably Drosophila fruit flies, their nervous system is quite different from that of mammals, with less similarity to humans. Moreover, relative to lower model organisms, the wide range of behaviours exhibited by mice (e.g. learning and memory, anxiety, social interactions) has a greater degree of similarity to human behaviours.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Before behavioural testing, mice will be handled to reduce the stress of human interactions. Behavioural tests in general do not cause pain and suffering, but there will be a limit on the total number of tests and the number of aversive stimuli given to any one animal. Tests involving electric shock will only be used at the end of the test battery if milder tests of learning and memory are either inappropriate or do not achieve the desired outcome. Moreover, mouse lines with visible neurological defects due to the genetic variant they harbour will not be subject to behavioural tests involving electric shock.

Good handling and injection technique will minimise any minor/brief distress associated with the systemic administration of test substances. Where multiple daily injections are given, the injection site will be changed to minimise pain and potential inflammation.

For surgical procedures, suitable anaesthesia and analgesia will be administered in consultation with the Named Veterinary Surgeon; any sign of suffering will be discussed with the NVS for immediate advice.

There are multiple established tests to assess seizure susceptibility in rodents. However, the methods that we have selected are the least invasive and most refined because each procedure (including an observation period) can be completed within 1 hour and is designed to induce seizures only in mice with an increased susceptibility to seizures, not in wild-type control animals. For example, in the water exposure test, the mouse is placed in a container of water that is sufficiently shallow to permit it to touch the floor with its hind

paws; consequently, unlike in the forced swim test (which is classified as severe), the mouse is not forced to swim.

Planned future work includes the use of home cage video-monitoring to assess the behaviour (including incidence of spontaneous seizures) of genetically altered mice, which may eventually lead to fewer behavioural tests being conducted in this project.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

To ensure experiments are conducted in the most refined way, best practice guidance in the following publications will be followed:

Handbook of Laboratory Animal Management and Welfare, Third Edition. September 2003. S. Wolfensohn & M. Lloyd. Blackwell Publishing Ltd. https://onlinelibrary.wiley.com/doi/book/10.1002/9780470751077

Guiding Principles for Behavioural Laboratory Animal Science. Edition One: November 2013. LASA. https://www.lasa.co.uk/wp-content/uploads/2018/05/LASA_BAP_BNA_ESSWAP_GP_Behavioural_LAS_Nov13.pdf

The Design and Statistical Analysis of Animal Experiments. March 2014. S.T. Bate & R.A. Clark. Cambridge University Press. https://doi.org/10.1017/CBO9781139344319

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The licence holder has a track-record in the application of 3Rs in their programme of work, including NC3Rs funding for a replacement strategy and sitting on an NC3Rs grant panel. To stay informed about new advances in the 3Rs relevant to this project, we will utilise the PubMed and NC3Rs websites. If refinements to experimental procedures are published that will improve animal welfare, we will conduct pilot studies to determine whether they are suitable for adoption in this project

169. Lymphocyte development and ageing

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

lymphocyte development, ageing, antibodies, gene regulation, signalling mechanisms

Animal types	Life stages
Mice	adult, juvenile, pregnant, embryo, neonate, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to understand gene expression and protein interactions that regulate development of B lymphocytes and antibody production, and how these change in ageing and disease.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

B lymphocytes generated in the bone marrow produce the enormous antibody repertoire that we need to fight millions of different infections we may encounter. We do not fully understand many of the gene regulation and signalling mechanisms required to produce sufficient numbers of B cells with sufficient quality of antibodies throughout our normal lifespan. Undertaking this work will help to reveal how B cells progress through multiple stages of development, and how they convert a few hundred antibody genes into millions

of different antibodies. This will help us to understand why some people fight infections well, while others do not, and may identify new therapeutic strategies that could be used.

More specifically, older people are less able to fight infection and to benefit from vaccination. This is partly because older people make fewer B cells in the bone marrow, and these B cells make fewer different antibodies. If we can understand the major pathways that are defective in ageing, we can design strategies to reduce these defects and boost the ageing immune system. This will make a vital contribution to improving healthspan (how many years we stay healthy) in our increasingly long-lived population. Unfortunately healthspan is not currently keeping pace with lifespan, resulting in an ageing population that is living longer with multiple health conditions that reduce quality of life. Finally these studies will also contribute to our understanding of immunodeficiency diseases caused by poor antibody responses, and to our understanding of B cell leukaemias that initiate in bone marrow B cells.

What outputs do you think you will see at the end of this project?

Using state of the art genome-wide sequencing and computational mapping of the DNA and RNA data, we expect to generate many large databases of information on expression of genes and antibodies and how they change in ageing. We will publish several papers on the three objectives of the project. We may identify new products for the clinic, or provide new knowledge on therapeutic combination of existing products. We may generate new intellectual property, which we will protect with patents. We will also engage in active dialogue with the public to inform people about our research and its relevance to ageing and healthspan.

Who or what will benefit from these outputs, and how?

The earliest beneficiaries will be researchers working in related aspects of lymphocyte development, who will benefit from our databases to inform their studies. They will benefit from new understanding of key cellular signalling pathways and gene regulation mechanisms that enable B cell growth and development, and how they are altered in ageing B cells.

Researchers working on ageing in other systems will benefit from new knowledge on common ageing pathways, and ageing heterogeneity (how individuals age differently)

Researchers working on growth, metabolism and gene regulation in other tissues will benefit from our findings, which will be relevant for many tissues.

In the longer term, clinicians and immunologists working with ageing patients will have greater understanding of the characteristics of the antibody repertoire that change with ageing, and may have new tools to assess immune system health.

Vaccinologists may have new insights into ways to boost vaccine efficacy, in the general population and in older people.

Clinicians treating B cell and other leukaemias will benefit from new understanding of how genes are organised on different chromosomes in the nucleus, and how this positioning may predispose to leukaemias.

Once intellectual property is protected, commercial interests may have new therapeutic products to develop.

How will you look to maximise the outputs of this work?

We will maximise the utility of the large datasets we will generate, eg by sharing data early and seeking collaborations on important discoveries that we do not have the expertise or resources to follow up. We will publish our research as preprints on bioRxiv in advance of submission to journals. We will publicise any unsuccessful approaches to the scientific community to avoid others wasting resources on these (eg published mouse models in which we discover a fault). We will present our results at scientific conferences before publication. We will secure any potential intellectual property by patent to protect UK commercial interests. We will do this early in order to avoid delays in publication. We will actively engage with appropriate commercial and clinical interests to progress our results towards the clinic in a timely fashion.

Species and numbers of animals expected to be used

- Mice: 7160

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice because the mouse immune system, in particular lymphocyte development, is extraordinarily similar to the human system. Furthermore, gene regulation and cytoplasmic signalling, our main investigation areas, are also very similar between mouse and human. Additionally individual genes can be effectively deleted in mice in specific tissues without affecting the whole animal. We will use two types of mouse models. First, we will study mice in which specific genes that we have shown are important in ageing, have been deleted only in B cells, ensuring the mice remain healthy overall. These studies will be in mice aged 3 months (equivalent to a human young adult). Second, we will compare 3 month-old wild-type mice with wild-type mice that we will age up to 24 months (equivalent to a human of over 70 years). This will enable us to study defects in B cells that occur as a consequence of ageing.

In addition to the fact that the ability to delete individual genes in mice enables highly specific and detailed studies that are not possible in humans, we are also particularly

restricted to using mouse models specifically for these studies, because, unlike human blood samples, it is very difficult to obtain human bone marrow samples for research.

Typically, what will be done to an animal used in your project?

A small number of female mice (<100) will be injected with hormones to increase production of embryos (superovulation) and a similarly small number (<50) will receive embryos via surgical or non- surgical procedures to establish new mouse strains. A very small number of male mice (<10) will be vasectomised to help with this.

For the vast majority of our studies, including both gene-deleted and aged mice, we will remove bone marrow and spleen from mice post-mortem, and purify and/or grow B cells from these samples. We will occasionally re-inject some of these purified B cells back into mice to determine how efficient they are at restoring B cells in the mouse.

What are the expected impacts and/or adverse effects for the animals during your project?

Superovulation, receipt of embryos and vasectomy described above are surgical procedures that are considered moderate, due to pain post-surgery. This is controlled by analgesia before and after surgery.

For most of the mice, we do not expect any adverse effects in our extremely high quality animal care facility. Some mice will be immunodeficient, but we know from experience that this has no adverse impact on health in our specific pathogen-free (SPF) facility. We also know that the vast majority of our aged mice remain healthy throughout the duration of our experiments. A small minority get tumours, but regular checking by our experienced animal technicians ensures these are detected early, and the mouse euthanised immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity for most of the mice will be mild. The expected severity for embryo recipients and vasectomy will be moderate. Together, the proportion of those in the moderate category will be 1% of the total mouse cohort.

What will happen to animals at the end of this project?

- Used in other projects
- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We need to use animals primarily because this is the only way that we can study the role of individual genes in detail, by investigating the effects of single gene deletion on all the stages of development they affect, in this case bone marrow B cell development. Furthermore, it is extremely difficult to study B cell development in the bone marrow in humans, because it is very difficult to obtain human bone marrow samples, in particular from healthy donors.

Nevertheless, as part of our current PPL and future plans, we have established a pilot human bone marrow culture system, which will replace some of our mouse studies and/or provide important cross species validation.

Which non-animal alternatives did you consider for use in this project?

We have considered using mouse bone-marrow derived B cell lines.

Why were they not suitable?

These lines only represent one stage of B cell development at a time, and thus we can't study how all the stages of development affect each other. Also the culture conditions they grow in do not allow all of the possible different antibodies to be made, and some cell lines only allow one type of antibody to be made, so we could not investigate the role of individual genes in generating the millions of different antibodies that our millions of B cells make.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have estimated the number of animals we will use from our previous studies using aged mice, and mice in which we have altered a gene. This has enabled us to predict the numbers of mice of the correct genotype that we will produce from breeding, and the numbers of aged mice that we will need.

Data from previous experiments has enabled us to estimate the numbers of cells we will need for each experiment, and consequently the numbers of mice of each genotype.

Important considerations have included adjusting for the reduced numbers of B cells produced by aged mice compared with young mice.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have consulted in depth with our Biostatistician, and have built on our previous experience of working with these and similar mice. In particular, we have learned that individual aged mice have much more variation in the genes they express compared with young mice. Accordingly we have had to increase the number of aged mice compared with young mice within some experiments, but this will reduce numbers in the future, since individual experiments will be statistically significant.

We have increased the focus of our experiments towards the B cell culture system that we have developed, instead of using ex vivo purified B cells. While culture systems have some caveats, and are not suitable for all experiments, they have the advantage that cell numbers can be expanded to provide many more cells per individual mouse, which also has the synergistic advantage that parallel experiments can be conducted in the same mouse rather than in separate mice, increasing statistical robustness. One mouse can provide the equivalent number of B cells expanded in culture to the number of ex vivo purified B cells from 20 mice. We have used the NC3R's Experimental Design Assistant to ensure we are considering all relevant aspects of design.

We will avail of ongoing improvements in next generation sequencing methods that have reduced cell numbers required. For example one of our methods previously required 10 million cells, but improved methods now only require half a million cells.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Our mouse facility has an extremely efficient pipeline of 10 day biopsies, and rapid genotyping by a commercial provider, that provides timely results to enable efficient breeding.

Selective deletion of individual genes in specific tissues will be achieved by the use of Cre-Lox recombination to delete the gene of interest in the desired type of cells. We will do pilot studies with cre recombinase expressing mouse lines crossed to mouse lines expressing conditional floxed genes of interest to ensure, first that the gene is efficiently deleted in the cells in which we want it to be deleted, and second that the approach does not delete the gene in other cells. For the latter we will make extensive use of genotyping for deleted genes, which has previously alerted us to off-target gene deletion events which necessitated re-design of our experiments. Pilot studies ensure minimum use of mice before decision points are reached in experimental design.

For our aged mouse studies, we have an established and highly successful programme of sharing of tissues with other investigators, which we will continue. This often includes up to 4 investigators using tissues from the same mouse.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use wild-type, transgenic, gene-targeted young mice up to 3 months, and aged mice up to 26 months.

Transgenic and gene-targeted mice usually only have a single gene, or at most, three genes, altered. Either by natural expression patterns, or by targeted deletion this only affects a small number of tissues. In this project these are primarily bone marrow and spleen B cells. Any potential pain or suffering would arise from infection, since these mice will have defective B cells and thus be more susceptible to infection. All mice are housed in very clean conditions, which prevent infections, and thus prevents any such pain or suffering.

Most experiments only take tissues post-mortem. A small number of experiments will involve injecting bone marrow cells into a recipient mouse to reconstitute the immune system. These experiments provide detailed in vivo knowledge on the ability of haematopoietic cells from a gene targeted mouse to contribute to B cell development, in ways that studying cells ex vivo can not do. Furthermore, relatively few cells from a single gene-targeted mouse can reconstitute several recipient mice, contributing to greater statistical power. In some cases these experiments will restore the immune system of immunodeficient mice, and will have no negative impact. In a small number of cases, wild-type mice will be irradiated to remove endogenous lymphocytes, to ensure donor bone marrow is providing reconstitution. This can occasionally lead to ill health, but these mice will be carefully monitored and any animal exhibiting pain or distress will be culled immediately.

Thus in most cases, there is no pain, suffering, distress, or lasting harm expected with the models or planned experiments. The exception to this is that a small number of animals that will be embryo recipients, or will be vasectomized, will experience transient pain due to surgery. This will be ameliorated by analgesia.

Why can’t you use animals that are less sentient?

We can't use animals that are less sentient, because they do not have an adaptive immune system that is comparable to the human system. This system first appears in evolution in the lamprey and zebrafish, but in a very rudimentary form, that is not suitable for in-depth studies. Species including C elegans and Drosophila melongaster, have rudimentary innate immune systems, but do not produce lymphocytes.

We also cannot use early stage mouse embryos because the adaptive immune system does not develop until late stages of embryogenesis.

Although some species including C elegans are established models of ageing, their lack of an adaptive immune system makes them unsuitable for our studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We are mindful of increased possibility of welfare costs as mice age, and we have implemented a programme of increased frequency of observation of these mice. We have developed a detailed checklist of possible age related changes, and procedures for monitoring and treatment, which informs our decisions. We will gather data from these studies to further refine best practice.

We will implement any refinements developed by our animal house staff, who have a long history of innovative practice, including environmental enrichment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow published best practice guidance from NC3Rs, LASA and the Home Office.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will stay informed about advances in the 3Rs during this project via the monthly NC3Rs e- newsletter, NC3R workshops and webinars, and our annual Institute 3Rs seminar. We will avail of Home office advice made available to us through our dedicated Home Office Liaison.

To implement advances effectively, we will follow advice from our local AWERB. Our animal facility also has a dedicated Strategy Committee and a User group, on which we are represented. Both groups discuss and make collective decisions about advances in the 3Rs and advise on how they can be implemented, both across the organisation, and by individual groups.

170. Gene and environment influences contributing to psychiatric disorders

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

addiction, impulsivity, zebrafish, cognition, behaviour

Animal types	Life stages
Zebra fish (Danio rerio)	embryo, neonate, juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to advance understanding of genetic and environmental factors that influence an individual's vulnerability to psychiatric disease, and the biological processes by which they act.

A secondary aim is to identify small molecules that ameliorate psychiatric disease phenotypes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Psychiatric or behavioural disease including addictions, anxiety disorders and depression is a major concern for modern society with huge financial and social cost: The World Health Organisation estimates untreated mental health problems account for 13% of the total global burden of disease. It is projected that, by 2030, mental health problems will be the leading cause of mortality and morbidity globally. The UK 2013 Chief Medical Officer's report estimated that the wider costs of mental health problems to the UK economy are £70–100 billion per year – 4.5% of gross domestic product.

Identification of genes and gene products influencing psychiatric disease phenotypes gives insight into the etiology of the disease allowing the identification of vulnerable individuals, the development of therapeutic interventions and more personalised therapies. Human genome wide association studies (GWAS) have identified a number of alleles and copy number variants associated with psychiatric disorders e.g.[Horwitz et al 2019 for review] but causality has yet to be established and the mechanisms by which these factors act, and which component behaviour they regulate, is in large part unknown.

Further, it is clear that environmental factors throughout the life course interact with genetic pre- disposition to influence behavioural outcomes (see Dick 2011 for review). Understanding the nature of these interactions and the biological processes affected will lead to better preventative and treatment options.

Horwitz, T., Lam, K., Chen, Y. et al. A decade in psychiatric GWAS research. Mol Psychiatry 24, 378– 389 (2019). https://doi.org/10.1038/s41380-018-0055-z

Dick, D.M. Gene-Environment Interaction in Psychological Traits and Disorders (2011). Annual Review of Clinical Psychology 2011 7:1, 383-409

What outputs do you think you will see at the end of this project?

Expected outputs include:

1) Identification of up to 20 genes affecting behavioural phenotypes associated with psychiatric disease. Specific phenotypes examined are; reward responses to drugs of abuse, impulse control, social interaction, anxiety, sleep, circadian rhythm, memory and learning.

2) Establishment of the developmental and cell biological processes by which genetic variants associated with psychiatric disease act.

3) Greater understanding of the interaction between genetic variants, developmental and environmental factors contributing to psychiatric disease.

3) 5 or more publications.

Who or what will benefit from these outputs, and how?

Long term benefits:

Academics, clinicians and the wider population:

Output 1, 2) Identification of genes and gene products influencing psychiatric disease phenotypes and how they act gives insight into the aetiology of the disease allowing the identification of vulnerable individuals, the development of therapeutic interventions and more personalised therapies.

Output 3) It is well established that environmental factors such as developmental stress, exposure to environmental toxins and dietary factors interact with genetic predisposition to influence the occurrence of psychiatric disease. Increased understanding of these interactions (for example, which genetic variant influences responses to specific environmental conditions) and how environmental factors impact on the aetiology and cell biology underlying behavioural disease will further the development of preventative strategies, of therapeutic interventions and of more personalised medicines.

Short term benefit: We use fish (zebrafish) as our model species. Zebrafish are vertebrate fish that are widely used as a model for the study of human development and disease due to the ease with which one can identify genetic factors that modify disease processes in these species. By extending the use of fish to the study of behavioural disease we contribute to the refinement of animal usage by reducing the use of mammalian species.

How will you look to maximise the outputs of this work?

To maximise outputs we aim to publish in well renowned journals, publicise findings via social media, the university Public Engagement office and at national and international conferences. We will collaborate with other researchers in the field, including industry, to ensure realisation of potential therapeutic benefits. Data will be stored in Institutional repositories (e.g. Apocrita) and made publicly available on publication in accordinance with Open Access policies. Identified lines will be shared with researchers on request.

Species and numbers of animals expected to be used

- Zebra fish (Danio rerio): Adult wild type or genetically modified fish: 10,000, wild-type or genetically modified larvae: 35,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is not possible to study complex behavioural processes such as those associated with psychiatric disease and addiction without the use of animals. Zebrafish are non-mammalian vertebrates that have been shown to have a translationally relevant behavioural repertoire as well as conserved drug reward response to addictive drugs.

Zebrafish have been extensively used as a genetic model for the study of development and thus a large number of resources including transgenic lines and mutants are available. Large scale screens are more feasible in these species than in rat or mouse. Thus, they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition.

Invertebrates such as Drosophila and C. elegans are other popular model systems for forward genetic screens. However, despite being useful models for the analysis of the acute effects of drugs of abuse, assays for psychiatric disease phenotypes and drug preference have yet to be established in these species. Furthermore, invertebrate brains do not show the same level of complexity as vertebrates and it is not clear whether the neuronal networks established to be involved in addiction and human behavioural disease are present in invertebrates. Thus findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

We use both larval and adult forms in our studies. Wherever possible, behavioural assays are conducted at larval stages. However, more complex behaviours associated with psychiatric disease, such as poor impulse control, deficits in social interaction, aggression, cognitive phenotypes, are not evident until juvenile or adult stages. In addition, many of the environmental factors that impact behaviour operate over an extended period or at juvenile stages requiring the use of older forms.

Typically, what will be done to an animal used in your project?

Wild type or genetically modified animals (fish carrying non-harmful reporter constructs or genetic mutations showing no clinical signs of harm) will be assessed for performance in behavioural assays. In the majority of cases fish will only be assessed in one behaviour during their lifetime. The exception to this may be evaluation of behaviour as larvae and subsequent evaluation of a second behaviour as adult. Behavioural assays in adults involve performance of operant learning tasks, such as approaching a light for food reward, as well as evaluation of natural behaviours such as response to a novel environment, startle responses, sleep, circadian rhythm or social interactions, and reward responses to commonly abused drugs. Larval behavioural assays are restricted to assessment of natural behaviours such as sleep, circadian rhythm, response to a novel environment, light or dark preference, or startle response.

Depending on the assay, behaviour may be assessed over a period of 5 min (response to novelty, social interaction), 1 hr (response to startle, reward response to drugs of abuse) or an hour a day over several weeks (operant learning tasks).

We assess the interaction between genetic variants and the environmental factor of developmental drug exposure.

What are the expected impacts and/or adverse effects for the animals during your project?

No adverse effects are expected from the behavioural assays themselves. The genetically modified lines used for our studies are not expected to cause harm; we predominantly assess behaviour in existing mutant lines that have no obvious morphological defects or clinical impairments. However, where a new line is generated injected constructs may cause developmental abnormality (25%). No animal showing abnormal development will be reared.

On occasion individual animals show increased anxiety, evident as rapid breathing and altered swimming behaviour, and fail to habituate to the behavioural assay equipment. Animals that exhibit such behaviour for more than 10 minutes on any one day are removed from study that day. If animals fail to habituate to the apparatus repeatedly over 3 days, they are not used further for behavioural studies but may be used for breeding or tissue-based studies.

Animals used in appetitive learning tasks for food reward may show weight loss over the course of the experiment (up to 10 weeks) if they fail to learn well. Where >10% weight loss is evident by visual comparison with non-experimental animals, fish are given additional food outside the training session.

Where the impact of drug exposure on behaviour is evaluated, exposure to drugs may cause an acute (<10min) avoidance response. On rare occasions, where genetic variants cause hypersensitivity, drugs may be toxic leading to abnormal swimming or haemorrhage. In this event animals will be immediately removed from the drug and killed.

Where fish are genotyped using finclip, fish may experience brief <24 hr post-operative pain. Swabbing (https://www.liebertpub.com/doi/10.1089/zeb.2016.1348) will be used as an alternative method for collection of DNA for genotyping wherever possible.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

> 95% of animals (larval or adult) are expected to be used in behavioural assays of mild severity. <5% of animals are expected to be used in assays of moderate severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to study complex behavioural processes such as those associated with psychiatric disease and addiction without the use of animals.

Invertebrates such as Drosophila and C.elegans are other popular model systems for forward genetic screens. However, despite being useful models for the analysis of the acute effects of drugs of abuse, assays for psychiatric disease phenotypes and drug preference have yet to be established in these species.

Futhermore, invertebrate brains do not show the same level of complexity as vertebrates and it is not clear whether the neuronal networks established to be involved in addiction and human behavioural disease are present in invertebrates. Thus findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

Which non-animal alternatives did you consider for use in this project?

We collaborate with human geneticists to examine the genetics of psychiatric disease based on analysis of human genetic datasets. Meta-analysis of pathways affected in human cohorts together with those identified from existing mutants and from pharmacological studies in fish (our lab as well as those of others) as well as other species is used to identify candidate genes and pathways underlying addiction and psychiatric disease.

Why were they not suitable?

Although human studies can identify genetic variants associated with disease phenotypes, the complex nature of human behaviour coupled with variance within human cohorts makes identification of significant associations difficult. Further, it is not possible to test causality using human subjects, nor to conduct cell biological studies to determine cellular processes affected. Previous animal studies have largely focused on candidates identified from human studies and cannot account for all the variance seen within human populations. In addition, most animal studies to date do not address the phenotypes outlined here.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Numbers are based on previous studies in our lab and the lab of others in the field and have been recalculated and checked using the NC3Rs Experimental Design Assistant tool.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use research experimental tools like the NC3Rs Experimental Design Assistant ensure appropriate study design and to help reduce the number of animals used.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

To minimise number of animals used we will optimise genotyping protocols so as to allow genotyping at early life stages to increase breeding efficiency. Pilot studies will also be undertaken for all new studies to help minimise animal usage. Wherever possible animals that are to be killed following behavioural analysis will be used to provide tissue for molecular studies and tissue will be shared. When conducting experiments to isolate phenotypic fish for genotyping to identify novel mutations affecting behaviour, computer modelling is used to predict the number of animals required.

To reduce the number of animals used we have established a collaboration with other large zebrafish institutes in the UK and the USA, whereby they provide adults or embryos from their mutagenesis screens and transgenesis programmes thus reducing the number of new mutagenized/ transgenic animals generated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We use zebrafish as our model species. Zebrafish are non-mammalian vertebrates that have been shown to have a translationally relevant behavioural repertoire and conserved drug reward response to addictive drugs. Thus they are the vertebrates with the lowest neurophysiological sensitivity likely to yield results relevant to the human condition.

Zebrafish have been extensively used as a genetic model for the study of development and thus a large number of resources including transgenic lines and mutants are available. Large scale screens are more feasible in these species than in rat or mouse.

Zebrafish ENU-mutagenised lines carry mutations at a greater density than mammalian mutagenised lines allowing more efficient screening of the genome (and therefore minimising the numbers of animals used). Zebrafish embryos and larvae are transparent allowing visualisation of morphological defects at early life stages minimising harm.

We primarily use observation of natural behaviours or appetitive learning paradigms as our means of assessing behavioural phenotypes. Behavioural assays of natural behaviours are not predicted to cause pain, suffering or lasting distress or harm. Appetitive paradigms can be considered less severe than those using aversive learning. Although on occasion we assess behaviour in response to aversive stimuli, in fish adverse stimuli are generally mild and short-lived, such as a single knock on the side of the tank, flash of light or sight of a predator.

Why can't you use animals that are less sentient?

Invertebrates such as Drosophila and C.elegans are other popular model systems for behavioural genetic studies. However, despite being useful models for the analysis of the acute effects of drugs of abuse, assays for psychiatric disease phenotypes and drug preference have yet to be established in these species.

Furthermore, invertebrate brains do not show the same level of complexity as vertebrates and it is not clear whether the neuronal networks established to be involved in addiction and human behavioural disease are present in invertebrates. Thus findings from Drosophila and C. elegans are less likely to be relevant to human biology than findings from studies on vertebrates such as zebrafish where the conservation of neuronal networks has been established.

We use both larval and adult forms in our studies. Wherever possible, behavioural assays are conducted at larval stages. However, more complex behaviours associated with psychiatric disease, such as poor impulse control, deficits in social interaction, aggression, cognitive decline, are not evident until juvenile or adult stages. In addition, many of the environmental factors that impact behaviour operate over an extended period or at juvenile stages requiring the use of older forms.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Refinements to minimise suffering include increased monitoring for all experimental animals, particularly following any invasive procedure (skin swabbing/anaethesia/finclip) or drug exposure. We regularly review protocols and the use of analgesics, for example post finclip, with the NACWO and NVS.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow FELASA and LASA best practice guidelines and the ARRIVE guidelines. In addition, I am a member of the EU zebrafish society (https://www.ezsociety.org/), a society dedicated to promoting and improving the use of zebrafish for biomedical research. Through active participation in this society I am party to EU wide best practice advice regarding the use of zebrafish for research.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We are kept up to date with advances in the 3RS through the NC3Rs newsletter direct to personal email addresses and via Institutional notification from the NACWO, compliance officer and licensed vet, and through the AWERB.

171. Studies on the neurobiology of sensation

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants
  - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
- Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

chronic pain, neuroscience, cancer, neuropathy, therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged
Rats	neonate, juvenile, adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The pivotal aim of this project is to determine the basic bodily processes that underlie the sensation of touch and pain, and how these processes are altered in persistent pain states. The ultimate objective is to translate pre-clinical observations to the clinical domain

in order that we can better treat chronic pain in patients through targeted pharmacotherapeutic application.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This work is important because pain remains a clinical unmet need with considerable socio-economic burdens because of the limited number and efficacy of existing therapies. Nearly 20% - that is >100m Europeans - suffer moderate or severe pain of long duration. Importantly, much of this pain is not adequately controlled by currently available pain-relief medications, and those therapies available are additionally associated with many significant and often dose-limiting side effects. There is clearly a pressing need for novel analgesic agents or therapies, and the effort to generate these will be greatly strengthened by a better understanding of the underlying mechanisms that cause and drive persistent pain. Chronic pain presents an intellectual challenge because of the complexity and range of biological and neuronal processes that change the way in which we perceive pain. Our work will examine the circuitry controlling and contributing to chronic pain and thus will increase our understanding of pain neurobiology, such that targeted pharmacotherapeutic approaches may be translated to the clinic.

Some studies of sensory phenomena are possible in humans however, mechanistic questions require invasive techniques that are not possible or feasible at present in humans and as such we propose the work herein.

What outputs do you think you will see at the end of this project?

We anticipate the following outputs:

1. Identifying novel pain mediators and analgesic strategies for varied pain states. It is likely that the precise role and involvement of these targets will differ depending on the precise pain state. We therefore require a variety of different models to investigate their function. Beyond new targets we are also working on novel analgesic strategies as to how persistent pain could be best treated.

2. Uncovering the role of neuro-immune interactions. We will investigate how nociceptors interact with immune and other supporting cells as these are frequently the source of pain inducing mediators.

3. Defining pain circuitry in health and disease. We will study how neuronal circuits transmit injury messages that lead to the sensation of pain, and how these circuits change in disease states. Fundamental questions regarding the integration of peripheral inputs with spinal circuitry and the top down cortical modulation of spinal circuitry will be answered.

4. Refining the administration of peri-operative analgesics to animals undergoing surgery to induce a chronic pain state. The positive impact of surgical administration of centrally-acting analgesics on the acute pain state (in the period immediately following surgery) is expected with no impact on the desired neuropharmacological experimental outcomes where the aim is to investigate chronic pain mechanisms.

Who or what will benefit from these outputs, and how?

1. **Immediate use of data and products**

This work will provide further knowledge of the normal properties and regulation of primary sensory systems and how they are affected in persistent pain states. Experimental results will be analysed and used to determine the direction and design of appropriate and efficient follow-up experiments. Data will also be written up and presented at international meetings and published in peer-reviewed journals for dissemination to the research community at large.

2. **Opening of new avenues for research**

We believe that the data generated from this project will open up new avenues of research. Research into and improved understanding of normal, pathophysiological and psychological pain processing and spinal cord function will lead to the determining of cell signals, neurotransmitters, channels etc. implicated in the production of pain syndromes. Each novel target identified will open a new avenue of research including replication and validation of hypothesis by numerous means.

3. **Practical Applications**

We believe that the work done in this project will have a future important practical value in the development of strategies to combat illness associated with chronic pain (such as arthritis, bone cancer, neuropathic pain). Previous studies indicate that the discoveries underlying chronic or persistent pain states in animals are transferable to analogous conditions in humans. In short, our hope is that our work will be translated into treatments for patients.

In total the knowledge that could arise from the proposed research would benefit many people within the pain community as we will significantly advance our understanding of the underlying pathology of pain conditions, particularly the recognition that chronic pain states are associated with maladaptive forms of plasticity in the peripheral and central nervous system.

How will you look to maximise the outputs of this work?

We have a number of external collaborators already in place (including those nationally in the UK as well as those internationally in Denmark, the USA and Canada). Understanding the neurobiology of sensation is of relevance to many audiences including academic and industrial researchers, clinicians, patients and the general public. Different forms of communication must reflect the varied nature of the beneficiaries and project results will be disseminated accordingly such that the importance of this research is described to different audiences in the most meaningful manner.

Results from this work will be presented at national and international scientific meetings with a focus on basic science and translational research. Data will be published in basic

science (pre-clinical and clinical) journals including those with an interdisciplinary focus. We will be targeting pain discovery and management communities. Social media such as the KCL pain-laboratories twitter feed will detail project progress, conference attendance and journal publication ensuring that the outputs of this work will be maximised.

Species and numbers of animals expected to be used

- Mice: 15400
- Rats: 3848

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We are principally interested in pain mechanisms. Some studies of sensory phenomena are possible in humans. However, the mechanistic questions that we wish to answer require more invasive techniques that are not possible or feasible at present in humans. In vitro techniques are also not sufficiently advanced (and are not likely to be so for some considerable time) that they can model the integrated actions of the nervous system. Thus, we will undertake some of our work in animals. The use of rats and mice throughout the project, which requires in some areas modelling of a pain state, is critically important. The neuroanatomy/neurophysiology of the rat and mouse is well understood and there is a comparable central nervous system complexity to the human. In rats and mice we can unequivocally establish which pathways underlie distinct pain processing mechanisms where a cell culture system would not model the necessary aspects of pain perception.

The questions we are asking usually relate to a specific human disease or pathology, which we need to model with suitable animal models: for instance TNF and NGF may interact in the context of interstitial cystitis, where there is an important inflammatory component, but not in the context of osteoarthritis.

Conversely, osteoclasts may play a role in sensitizing neurons in osteoarthritis, but obviously not in bladder pain. We therefore need to study a range of animal models of different human diseases.

Some of these animal models are short onset and short duration (hours) and can therefore be studied acutely in animals. For example, the injection of a chemical agent like capsaicin in the hind paw is associated with a nocifensive reflex and paw withdrawal from a noxious stimulus which are observed and recorded for up to 120 minutes. Other models, like their human counterparts, develop and change over time and so some of these experiments may last weeks or even months. For example, the injection of the chemical monoiodoacetate in the knee as a model of osteoarthritis is associated with nocifensive reflex and paw withdrawal from a noxious stimulus which are observed and recorded for

several weeks. The prolonged time course of some experiments and the fact that one of the most important outcome measures in our work is behavioural assessment of the animal, means that only some work can be done on animals under terminal anaesthesia - the remainder will require the use of recovery protocols.

Our scientific questions largely relate to the study of pain mechanisms in mature animals. However, in some cases an intervention is necessary at an earlier developmental stage. One example is the use of systemic capsaicin or resiniferotoxin, which if given in the early post-natal period (but not later in life) in rodents leads to a permanent depletion of nociceptors. This provides an important control experiment in mature animals. Another example relates to epigenetic studies. There is a belief that early life experiences may have a long-term regulatory role function of pain sensitivity, explaining why some patients appear to be very vulnerable to develop chronic pain. To study this phenomenon, it is necessary to give an intervention, such as an inflammatory stimulus or an inflammatory mediator to developing animals, even though the major aim is to understand the consequences for the mature animal.

Typically, what will be done to an animal used in your project?

Typically our experimental themes (in terms of what will be done to an animal used in this project) will rely on the following sequence steps following determination that animal work is necessary for the question at hand:

1. Genetically label or manipulate the cell population under study, e.g. inject AAV9-GCaMP6s virus into a rat to label DRG neurons or breed a transgenic mouse line which is knockout for a putative pain mediator

2. Induce a pain model

3. Apply a treatment (either in the form of a drug or toxin or using neuromodulation)

4. Test the functional outcomes of this treatment through the means below:

a) via behavioural studies: we have begun to innovate with assays that capture more natural, spontaneous animal behaviour – e.g. using home-cage monitoring. We will undertake a variety of behavioural tests of pain sensitivity. Most use threshold stimuli (eg heating the hindpaw) and the animal is free to withdraw from the stimulus at any time. Two, however, use surprathreshold stimuli (the formalin test and the capsaicin test).

b).by measuring neuronal activity in real time, using terminal electrophysiology, terminal calcium or optical imaging or – only when absolutely necessary for the scientific question: optical imaging or neurostimulation in awake animals (protocols 3-7).

5. Sacrifice the animal through schedule 1 or perfusion for subsequent anatomical studies or gene expression/protein assays.

What are the expected impacts and/or adverse effects for the animals during your project?

For all protocols: In the event of reaching the end-points described below, humane killing of the animal would be carried out by a schedule 1 method.

Weight: Weight loss of less than 10% in the absence of other clinical signs (see below) will result in increased monitoring and supportive measures such as wet mash diet. Weight loss of 15% in the presence of other clinical signs (see below) will result in humane killing. Weight loss of 20% in the absence of any other clinical signs will result in humane killing.

Posture: Hunched posture is indicative of discomfort, distress and pain. Continuous hunched posture or repeated phases of intermittent hunched posture over a period of 24h or prostration will result in humane killing.

Coat: Changes in animal's coat and condition is indicative of discomfort, distress and pain. Animals with a staring coat with marked piloerection will be humanely killed.

Behaviour: Rodents tend to be curious, exploratory and sociable in behaviour. Subdued behaviour and isolation are indicative of discomfort, pain and distress. An animal showing subdued behaviour, even when provoked, and little peer interaction, will be humanely killed. Similarly, animals which exhibit vocalisation to handling or gentle touch for more than a transient period will be humanly killed.

In addition the following expected impacts and/or adverse effects are noteworthy:

1. **Regarding new mouse lines:** It is not possible to fully predict the nature or severity of any potential defect and for all types of mice. As a result, with new lines, there will be careful monitoring for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed, or in the case of individual animals of particular scientific interest, advice will be sought from the Home Office Inspector. Where the immune status of the animals might compromise health, they will be maintained in a barrier environment. Small samples of tissue will be taken for genotyping. No more than 0.3 cm of the tail will be removed. Other than in terminally anaesthetised animals, dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm.

2. **Regarding dosing of agents:** Other than in terminally anaesthetised animals, dosing of agents will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than transient discomfort and no lasting harm. The dose of each agent will be chosen to avoid adverse systemic effects with veterinary consultation where appropriate, for novel agents or combinations thereof. As the majority of agents administered are produced to human clinical standards and their dosages have already been defined in animal models, adverse events are less likely to occur. In our experience, animals rarely die post i.v or i.p. injection (less than 1%) and generally recover well post anaesthesia. Dosing regimens for therapeutic agents is likely to be known in most cases. If dose escalation studies are required, they will be performed such that animal behaviour post administration can be observed for at least 15min before proceeding to the next higher dose (in a different animal). Thereby any acute adverse

effects can be detected. Control/vehicle substances will be administered first. If animals do not recover from acute adverse effects by the end of the working day OR show signs of pain exceeding a moderate severity they will be killed by a schedule 1 method. Some agents may cause adverse effects such as diarrhoea, skin and coat changes, feeding and behavioural abnormalities. We will regularly check the animals for limiting clinical. If any limiting clinical sign (see end points described above) is more than transient then the animal will first be closely monitored and then humanely killed by a schedule 1 method or anaesthetic overdose if there is no improvement within 48h.

3. Labelling of cell populations: In rare instances, surgeries to administer viruses/labelling agents may have complications associated with the procedure: i. Deaths resulting from anaesthetic or surgical complications are most uncommon (<1%) and will be minimised by ensuring correct dose/body weight and maintenance of body temperature (e.g. by use of warming pads). ii. Post-surgical infections are rare with the procedures described here. The risk of infection or delayed healing will be minimised by good surgical and aseptic techniques. Any signs of wound infection will be treated with antibiotics and wound powder, and if signs persist for more than 48 h the animal will be killed by a schedule 1 method. iii. Skin closure can produce local pain and/or necrosis and the risk will be minimised by removing any non- resorbable closure devices (e.g. stitches, clips) at approximately 7-10 days post-surgery. Reopening of wounds is a rare occurrence, but if it did occur, then it will be treated by re-closure under short-term anaesthesia on one occasion only. iv. Any animal showing signs of uncontrolled pain (described in 'Monitoring of animal characteristics and end-points' below) will be humanely killed. v. The injection of viruses into deeper brain regions, particularly into the parabrachial area may result in vestibular problems due to the proximity of the target to the vestibular nuclei. Vestibular problems, including some gait abnormality, turning upon picking up by the tail and escape upon touch always occurring in one direction, shall lead to termination of the experiment. In any such cases, tissue from these animals may be taken after perfusion with fixatives or under deep anaesthesia or after killing by a schedule 1 method.

4. Behavioural testing: Very few adverse events are anticipated with most of the behavioural tests we use. Most use threshold stimuli and a cut-off, preventing unintended adverse events. Some cold and heat suprathreshold tests induce a brief and transient painful episode, but no adverse effects are expected beyond that. In the very unlikely event that during a test an animal shows clear signs of distress (abnormal or extreme response to the test stimulus, aggression or unresponsiveness), it will be removed from that test and not entered into any others. In these tests animals will generally be habituated to the testing environment prior to the start of the experiment. The formalin test induces two phases of pain-related behaviour. We are evaluating a refined procedure using volatile anaesthetics to reduce or eliminate the first, and more intense, phase while leaving the second phase intact. If this proves fit for purpose it will be adopted in all experiments. If animals show unexpected severe reactions to capsaicin we will use local cooling of the treated paw (using cool water) to ameliorate the effects of capsaicin

5. Anaesthesia: A small number of animals may die from respiratory depression (1-2 %) and/or hypothermia, evidenced by depression in rate and rhythm of respiration and cold/pale extremities. To mitigate these effects depth of anaesthesia will be monitored regularly using the following checks: a) testing the pedal reflex, b) checking the rate and rhythm of respiration. Heating will be provided during anaesthesia where possible. For prolonged periods of anaesthesia, animals will be periodically rehydrated by subcutaneous injection of appropriate fluids (preferably glucose or dextrose/saline), as necessary. Animals receiving inhalation anaesthesia will, where possible, be allowed to recover in an induction box filled with 100% medical air or air, the colour of mucous membranes the depth and rate of breathing and pO2 would be monitored. The frequency of anaesthesia inductions will be kept to a minimum whilst still achieving the scientific objectives. Multiple procedures will be combined, where possible, to minimise frequency of induction. It is expected that at least 90% of the animals will receive no more than 3 general anaesthetics during the experiment. Between induction sessions, animals will be fully recovered, eating and drinking before being re-anaesthetised. Animals will not be re- anaesthetised if they have not resumed normal mobility and eating and drinking.

6. Terminal procedures: The terminal experiments measuring activity in sensory systems are not expected to have any adverse consequences. A surgical level of anaesthesia will be maintained throughout. Physiological functions such as deep body temperature, blood, pressure, end-expired CO2 will be monitored and maintained within normal limits. If a power failure occurs while an anaesthetised animal is being artificially respired, the animal will be killed by anaesthetic overdose.

7. Blood Sampling: Blood sampling will be undertaken under anaesthesia. Adverse effects are expected to result in no more than transient discomfort (after recovery from anaesthesia) and no lasting harm. Any bleeding after blood sampling (from superficial veins) will be controlled by local pressure and/or by application of a haemostat. If bleeding cannot be controlled the animal will be killed by a Schedule 1 method or by anaesthetic overdose.

8. Long term spinal stimulation studies: Certain complications may arise as a result of implantation surgery or the animals having connectors fixed to the skull and being physically connected to a stimulation device. Following epidural electrode implantation, animals will be monitored for 3-5 days for signs of hind limb dysfunction, spinal asymmetry, difficulties in movement, or signs of pain or major discomfort, before being considered for a study. Any animals showing these signs will be humanely killed. If post-implantation motor impairment is detected, such that the animal is unable to groom or move easily to reach food or water, it will be humanely killed. Partial evulsion of the stimulating lead near the point of connection to the skull-mounted pedestal may occur due to the animal's normal grooming. In this case, the animal will immediately be assessed to determine whether repair is possible. If this is deemed to be practical, the animal will immediately be re-anaesthetised and the lead replaced beneath the skin. Such a repair will be carried out only once. Should the problem recur, the animal will either be killed by a Schedule 1 method or anaesthetised and subjected to a terminal experiment. Partial

dislocation of the skull-mounted connector pedestal may occur due to failure of the cement-screw bond. In this case, the animal will immediately be assessed to determine whether repair is possible. If this is deemed to be practical, the animal will immediately be re-anaesthetised and bond repaired. Such a repair will be carried out only once. Should the problem recur, the animal will either be killed by a Schedule 1 method or anaesthetised and subjected to a terminal experiment. We will also observe on a daily basis the general behaviour and condition of animals during the periods of chronic sub-threshold stimulation and will we adopt the procedures and endpoints described. In the event that signs of distress are observed during the chronic stimulation period, we will pause the stimulation and reassess the current threshold. Regardless of whether or not this has changed, we will consider reducing the chronic stimulation current to alleviate the distress. We may cease stimulation completely for a period to see if distress signs are alleviated. If distress signs return when stimulation is restarted at the original (or lower) level, we may reduce the current further. In the unlikely event that minimal current intensity (0.1mA) still causes signs of distress, the experiment will be terminated and the animal either subject to a non-recovery electrophysiological experiment under general anaesthesia or killed by a Schedule 1 method. Tissue samples may be recovered from these animals. Any other adverse effects will be carefully documented and reported to BSU staff.

9. Neuropathy studies: Pain in the affected limb is the main intended adverse event. It usually manifests in a slight guarding of the paw. Up to one week following neuropathy, a mild motor impairment may also be observed (dragging of the hind limb). This will be permitted, but thereafter the animals should exhibit weight bearing on all four paws and a normal locomotor rhythm – albeit retain an eccentric paw placement (guarding). Rarely (<0.1%) an animal may show autotomy following nerve injury; if loss of more than two phalanges of two digits occurs the animal will be killed. Any animals exceeding a moderate level of pain will be culled as described in the section ‘Monitoring animal characteristics and end-points’, below. Following epidural electrode implantation, animals will be monitored during recovery until they are fully conscious. After recovery, animals will be monitored for 3- 5 days for signs of hind limb dysfunction, spinal asymmetry, difficulties in movement, or signs of pain or major discomfort, before being considered for a study. Any animals showing these signs will be humanely killed.

9. Drug induced neuropathy studies: Pain in the extremities is the main intended adverse event following induction of chemotherapy-induced neuropathy. However, when not specifically tested, the animals generally do not display any obvious outward signs of pain. Initial weight loss and general malaise may be observed while injections are ongoing and chemotherapeutic agents are taking their maximal effect. Animals will be carefully monitored and weighed every day while injections are ongoing, and regularly thereafter (min 3x a week), once pre-injection weights have been recovered. We will follow the guidelines and end-points set out in ‘monitoring animal characteristics and end-points’, below. Rarely (<0.1%) an animal may show autotomy following retraction of nerves as a result of the neuropathy; if loss of more than two phalanges of two digits occurs the animal will be killed.

10. Cancer pain studies: Subsequent to intraosseous injection of tumour cells, animals can show signs of lameness within hours. If animals do not recover normal mobility within the working day or within 24h (whichever is earliest), they will be culled by Schedule 1 method. Progression of bone tumours and model-specific endpoints: The prime method of tumour monitoring will be by looking for clinical signs including distress/pain, changes in mobility and abnormal characteristics (listed in section 'monitoring animal characteristics and endpoints' below). In some cases longitudinal imaging may be suitable to assess tumour progression. One endpoint will be the appearance of a visible surface tumour on the hind limb, the animal will be killed by a humane method. Impediment of locomotion due to tumour growth in bones/joints is likely, particularly after intratibial injection and at later progression stages. If an animal shows distress/pain reaching moderate severity limits OR exhibits non- weightbearing lameness, it will be killed by a humane method.

11. Induction of inflammation: Most of the experiments to be performed under this protocol will be performed on entirely on anaesthetised preparations and no animal welfare issues associated with the models is anticipated. The studies performed on conscious animals under this protocol will persist for relatively short time periods and few adverse effects beyond pain are not expected. All studies done on conscious animals should only induce pain at the affected site and not exceed moderate levels of pain as described in the section below 'monitoring animal characteristics and endpoints'. If these limits are exceeded the animals will be humanely killed. Systemic administration of LPS may cause hypothermia, hypotension, diarrhoea or lethargy. These may be expected to last for up to 4 hours and then subside; a saline injection will be given to these animals to ensure adequate hydration. UV irradiation: UV irradiation will produce inflammation of the paw which may include some sloughing of the irradiated epidermis. If this occurs and there are subsequent signs of infection, the animals will be humanely killed. Animals do not usually avoid weight bearing of the treated paw. However, all animals will be observed for signs of excessive discomfort such as sustained licking of the paw and other signs of distress. In those cases, they will be killed humanely.

12. Induction of chronic inflammation: For all types of chronic inflammation, pain in, and swelling of, the affected site is the main intended adverse event. However, pain should not extend beyond the affected limb or exceed a moderate level. To this end we will adopt the criteria and endpoints described in 'monitoring animal characteristics and endpoints' below. UV irradiation: UV irradiation produces an inflammatory response that typically grows over 48hours and then subsides over the following few days. If irradiated animals show signs of increasing inflammation beyond 72hours, they will be humanely killed. UV irradiation may induce some sloughing of the irradiated epidermis. If this occurs and there are subsequent signs of infection, the animals will be humanely killed. Animals do not usually avoid weight bearing of the treated paw. However, all animals will be observed for signs of excessive discomfort such as sustained licking of the paw and other signs of distress. In those cases, they will be killed humanely. Administration of inflammatory mediators into the CNS may result in systemic signs of an inflammatory

reaction such as changes in core temperature, sedation or lethargy, hypotension, and loss of body weight. All animals will be monitored for up to 6 hours on the day of injection, and daily thereafter for signs of ill-health. Any animal not recovering within 48h will be humanly killed. Injections of CFA or other irritants subcutaneously or into a joint are expected to cause local oedema and swelling and often some erythema. These signs typically increase in the hours following treatment and reach a stable plateau in the case of CFA with a few days. The local inflammation subsides, depending on CFA dose, after a week or two. Animals will therefore be monitored daily for 3 days and thereafter at least every other day, for signs of excessive local inflammation or the presence of widespread inflammation, and if detected, those animals will be humanely killed. If there are clear signs of irritation such as persistent scratching causing tissue damage the animal will be killed by a schedule 1 method. Some weight bearing of a treated limb is expected and if animals show absence of weight bearing persisting for more than 24 hours, they will be humanely killed. The inflammatory agents may in some animals induce diarrhoea. If symptoms persist for more than 24hours the advice of the NVS will be sort.

13. Induction of osteoarthritis: The surgical models of osteoarthritis typically develop slowly over many weeks. In contrast, the chemical methods of inducing the condition typically have a short lasting (days) inflammatory response which is sensitive to non-steroidal anti-inflammatory (NSAID) drugs, followed by a persistent phase of hypersensitivity relatively insensitive to NSAIDS. Signs of pain – increased sensitivity to local stimuli and reduced weight bearing of the treated limb - develop slowly in the surgical models and rapidly in the chemical models. Animals will therefore be monitored daily for the first 3 days and thereafter weekly for signs of excessive adverse effects. If weight bearing of a limb is lost for more than 24 hours, those animals will be humanely killed. Osteoarthritis may increase joint instability and in rare cases may lead to skeletal deformations. Any animals unable to normally reach food and water will be humanly killed.

14. Brainstem mechanisms underlying neuropathy/cancer pain: Animals undergoing surgical procedures where no peri-operative analgesia is given may have additional pain-like responses including prolonged vocalisation upon handling, a higher incidence of autotomy and more than 10% weight loss. All factors will be end points. Animals will be under daily surveillance in the acute post- operative 5 day period in order that, if these end points are reached, the animals may be culled to reduce suffering.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Protocol 1: Mild severity (10000 mice)
- Protocol 2: Moderate severity (2000 mice 2000 rats)
- Protocol 3: Moderate severity (1000 mice 1000 rats)
- Protocol 4: Moderate severity (400 mice 300 rats)
- Protocol 5: Moderate severity (1000 mice 1000 rats)

- Protocol 6: Moderate severity (500 mice 500 rats)
- Protocol 7: Moderate severity (500 mice 500 rats)
- Protocol 8: Moderate severity (16 rats)
- Protocol 9: Moderate severity (32 rats)

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Some studies of sensory phenomena are possible in humans however, mechanistic questions require invasive techniques that are not possible or feasible at present in humans and as such we propose the work herein. We are principally interested in pain mechanisms. In vitro techniques are also not sufficiently advanced (and are not likely to be so for some considerable time) that they can model the integrated actions of the nervous system. Thus, we will undertake some of our work in animals. The use of rats and mice throughout the project, which requires in some areas modelling of a pain state, is critically important. The neuroanatomy/neurophysiology of the rat and mouse is well understood and there is a comparable central nervous system complexity to the human. In rats and mice we can unequivocally establish which pathways underlie distinct pain processing mechanisms where a cell culture system would not model the necessary aspects of pain perception.

Which non-animal alternatives did you consider for use in this project?

It was not an option to consider non animal alternatives for use in this project.

Why were they not suitable?

The functionality of the circuitries that we wish to investigate, which rely on peripheral and central nervous system processes as peripheral and central manifestations of pain impact one another, cannot be investigated in vitro and would be too complex to investigate other than in vivo .

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used will vary from procedure to procedure depending on the degree of variability in the experimental measures, but our extensive experience with these models has shown that group sizes of 6 – 8 (rats) and 8-10 (mice) are generally appropriate. For those procedures involving surgery it is scientifically more rigorous to include sham operated control animals in an experiment. However, for techniques which are well established and for which we know from experience that there is no sham effect we will not include such animals in every experiment, but refer to historical control data. The effect of sham surgery will be reviewed periodically and when a new experimenter is using the technique.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

I am familiar with the NC3Rs Experimental Design Assistant having used it for my NC3Rs studentship application which regards refinement in the use of peri-operative analgesia in generating animal models of chronic pain and for all subsequent studies using animals. The use of this tool when planning experiments means that the numbers of animals being used in the project is at a minimum.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will seek to reduce the number of animals studied by careful experimental design, the adoption of sensitive outcome measures with small variation and the study of only the most relevant time points. Where possible each animal will be used as its own control. In all in vivo tests the number of animals in each group will be the minimum required to allow valid statistical analysis. It is possible in some instances to supplement the in vivo work outlined by using in vitro cell systems to analyse, for example, the effect of inflammatory mediators on receptor or ion channel expression and we plan to use such studies extensively. However, it is not yet possible to make stable cell lines of sensory neurones, our main cell type of interest, or to mimic the long-term effects of neuronal damage or inflammation that occur in vivo. In these instances, the use of animals is unavoidable. By cryopreserving sperm we will reduce the number of animals culled in breeding programmes.

Several of our protocols involve treating one hindpaw in some way, for instance delivering a neuronal labelling compound intraplantarly. Conventionally, a separate cohort of animals is used as controls.

However, where we know the control intervention is non-noxious, we will also use the contralateral hindpaw as a control.

To further reduce the numbers of animals used and to refine animal use wherever possible, animals may be tested within the same protocol with additional compounds. This may occur with protocols where extensive surgery is required to establish the baseline state (e.g. peripheral neuropathy) and the risk-benefit assessment is arguably in favour of reducing the number of animals submitted for surgery rather than reducing the number of compounds and behavioural tests that an individual animal is subjected to. All compounds will not be tested in all in vivo models but rather will be initially examined in the model most appropriate for that class of compound in which activity could be anticipated.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

During this project we will use animal models of neuropathy, cancer pain and inflammatory pain. The methods that we will apply include cell population labelling, behavioural assays and electrophysiology techniques. The extensive use of rodents in biological research has already provided considerable information on nociceptive and inflammatory processes. We will limit the severity of our models by limiting the time for which animals are kept following surgery or induction of inflammation as much as possible.

We will also be working on a specific example of refinement with our NC3Rs funding, where we aim to refine the common procedure of administering no or limited peri-operative analgesia to animals undergoing surgery to induce a chronic pain state.

None of our protocols fall under the severe category.

Why can’t you use animals that are less sentient?

Due to the nature of our studies, which aim to identify the mechanisms that underlie the progression of chronic pain states, using animals that are at a less sentient life stage is not possible since the diseases that we study are progressive. Pain can only be properly understood via the integration of knowledge spanning molecular, cellular, systems and psychological levels, hence less sentient species are not suitable. For a proportion of the animals that we use, following behavioural and/or electrophysiological experiments, the

study will proceed in the terminally anaesthetised animal as ex vivo tissue is harvested for subsequent immunohistochemical analysis for example.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For the majority of procedures (some exemptions sought within), animals will be provided with suitable post-operative analgesia. For establishment of models, suitable post-operative analgesia will be provided in a manner that does not affect disease progression. All will be performed under veterinary guidance. Animals will be regularly monitored for post-operative recovery. In addition animals may be trained to self-administer analgesics.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

No answer provided

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Since the NC3Rs are one of my funders, I participate in RSPCA and NC3Rs meetings. This allows me to keep informed about advances in the 3Rs, and implement any advances possible to my projects.

172. Production of genetically altered animals and provision of tissues (service provision)

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Transgenics, Cryopreservation, Genetic Alteration

Animal types	Life stages
Mice	adult, neonate, juvenile, embryo, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To supply genetically altered (GA) mice for scientific research, allowing researchers to advance our understanding of health and disease. This PPL allows us to freeze mouse lines for long term storage, to bring in mouse lines established elsewhere, and to generate novel lines in house.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The biological, physiological and genomic similarities between mouse and human have informed research into human disease for decades. The generation of complex genetically modified mouse lines not only affords better mechanistic understanding of natural biological processes and how these go wrong, but also enables the development of

therapeutic interventions (where appropriate). Exploitation of cutting-edge genome manipulation technologies allows the generation of models that better recapitulate normal and diseased states, thus generating more actionable knowledge 'per animal'.

Separately, having a centralised cryopreservation and rederivation program in our state-of-the-art facility ensures the minimum number of live animals at any one time and the provision of high-quality pathogen free (SPF) GA animals for research for the Institute and the wider scientific community.

What outputs do you think you will see at the end of this project?

Genetically Altered (GA) mice:

By ensuring the Transgenic Service remains a centralised core service we can ensure that each technique is only performed by specially-trained, highly-skilled staff, resulting in efficient animal production of high quality animals.

Centralisation of expertise allows new procedures and techniques to be adopted and benchmarked efficiently, ensuring mouse production approaches are state-of-the-art. Further, centralisation in transgenic production and also avoids the need for individual Project Licenses to require those procedures. Importantly, a centralised service can have a profound improvement on the efficiency of transgenic production, reduction of mouse usage and consistently improved welfare standards.

In addition, a rederivation program in our state-of-the-art facility, by our highly-skilled team will provide to the scientific community high quality SPF GA animals

New information/Publications:

New findings will be disseminated locally via the AWERB, User Groups, Animal Facility and seminars. Externally, these findings will be disseminated by peer reviewed publication and seminars.

Who or what will benefit from these outputs, and how?

The immediate beneficiaries of this GA service provision will be the researchers, and the advancement of the biological questions answered by the generation of GA animals. In the longer term it is hoped that this will translate into advancement of understanding of health and disease, ultimately enabling novel therapies.

The Transgenic facility acts as a core facility for GA mouse production, breeding, rederivation and cryopreservation, for local academic community. The centralisation of transgenic production into a core facility allows standardisation of procedures, consistency of approach, adherence to regulation, balance of harms and benefits, rigorous quality control/assurance and reproducibility. Further, we have developed robust pre-experimental workflows that ensure suitability of the model, compliance (on destination PPL), genotyping protocols, funding structures and a clear delineation of roles and

responsibilities. The end result of these processes is high quality, efficient production of GAAs that are tailored to the researchers needs.

How will you look to maximise the outputs of this work?

We will ensure that all the new technologies discovered by transgenic team, that will lead to a more efficient and finer generation of GA animals will be exchanged within the scientific community by: being published in peer-reviewed journals, sharing our knowledge through scientific conferences and meetings, submitting posters in the conferences with our new discoveries in the transgenic field. In addition, as a member of the International Society for Transgenic Technologies (ISTT) I am always available to answer relative questions through their internal email ISTT list. This will include the sharing of both successes and failures, lending advice to colleagues, and receiving advice from peers.

Species and numbers of animals expected to be used

- Mice: 15000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are widely used to gain an understanding of the complex cellular interactions that underpin both health and disease. Mice are well adapted to the laboratory setting, their small size makes it permissible to house multiple animals in a space and cost-efficient manner, and they are generally easy to handle and dose. Much of its biology and biochemistry mirrors that of human, and the mouse genome has been well characterised.

The life stages of mouse that will be used in this license are: adult, neonate, juvenile and embryo. We need to use the life stages that best address the biological questions that are posed by researchers. In addition, some technologies of genomic manipulation require specific stages of mouse development.

Typically, what will be done to an animal used in your project?

In the generation of a GA animal the following procedure will be implemented:

All putative projects to generate a novel mouse line are subjected to a pre-experimental design meeting where the appropriateness of the model and the need to generate a new line are assessed. Also addressed are the legal, regulatory, funding, technical and logistical aspects of the project.

Following a successful design meeting, optimal experimental parameters will be assessed in vitro (ie without generating animals). The goal of the in vitro optimisation is to both

assess the fidelity of the experimental approach (eg is the approach technically sound) but also to give an indication of the efficiency of the approach to allow us to gain an understanding of the total number of animals we need to generate to ensure project success. Importantly, in the instance that we wish to explore a new approach or technique this will first be performed in vitro where possible. These experiments will typically be short term (2-4 days), using a minimal number of embryos to quantitatively assess the efficiency.

After we understand the efficiency of the given approach, we will use this information to ensure the minimum number of donor animals are used to provide an appropriate yield of embryos for manipulation (microinjection – electroporation – rederivation – cryopreservation) (see general constraints). The optimised conditions will be used to make the desired genetic alteration in the embryo's DNA, embryo health will be assessed, and then embryos will be surgically implanted (see general constraints) into foster mice. Note – some embryos may be kept in culture for further experimentation or may cryopreserved for future study/use.

For approaches in which genome alteration was performed in the embryo, the resultant pups will be mosaic (different edits in different cells of the animal). For approaches where genome manipulation was performed in embryonic stem (ES) cells, the resultant mice will be chimeric (consisting of both ES- derived and donor-derived cells). Both mosaicism and chimerism is then 'purified' through breeding to generate pure GA lines. The new lines will be monitored by a team of scientists and technicians to ensure Germ Line Transmission (GLT) is obtained in a time sensitive manner and eventually will be transferred to the end user for further studies. All the surrogate mothers will be culled after the weaning of pups. All the pups produced that have undesirable genome manipulation will be made available for training purposes or will be culled.

What are the expected impacts and/or adverse effects for the animals during your project?

From our experience through the past 15 years, the vast majority of animals will experience no or limited adverse effects (eg transient pain from injections of hormones). In protocols that involve surgical procedures, analgesia and anaesthesia will be used to limit pain to the animal that results from these procedures (surgical procedures/administration of pain relief, see general constraints). Any pain associated with surgery is not anticipated to last more than a few hours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity of the animals used at this PPL, will be approximately 51% mild such as hormone dosing, 29% sub-threshold for transgenic breeding, and others approximately at

20%, that will be used as embryo recipients and vasectomized males will experience a moderate severity.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Because the aim of this PPL is to create, import/export, breed and maintain genetically altered (GA) mice and supply them for scientific research. In order to understand the maintenance of health and the development of disease (for example cancer), it is necessary to use animal model systems that can authentically recapitulate the complex cellular interactions involved.

Which non-animal alternatives did you consider for use in this project?

- None.

Why were they not suitable?

The aim of this license is to create GA animals

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Over the last five-year period (excluding 2020, due to covid-19), both the average number of experiments per year performed in the genome editing core and the average number of animals per experiment were approximatively equal to 50. Assuming a 20% increase in demand due to the covid backlog, the predicted number of animals required during the 5-year time period covered by this PPL equals 5*(50^2)*1.2 = 15000 animals.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Unnecessary production of genetically altered animals will be avoided by searching cryobanks and databases. Examples of resources available include:

Mouse locator: https://bioinformatics.crick.ac.uk/mouse_locator/

NC3R's mouse database: http://www.nc3rs.org.uk/category.asp?catID=8 Jackson laboratory: http://www.jax.org/ http://jaxmice.jax.org/index.html Cre transgenic database: http://www.mshri.on.ca/nagy/Cre-pub.html

The strain used for generating a new colony will be carefully considered to avoid producing unwanted mice. Animals will only be bred if a user requirement has been established, and the breeding programme will be subject to regular review to optimally meet anticipated demand. Spare animals will be made available for use on other scientific projects.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We take an array of measures to optimise the number of animals per project/experiment:

- First and foremost, we have pre-experimental design meetings that address the regulatory, scientific merits, experimental and quantitative aspects of the project.

- We assess in vitro, through pilot experiments, the efficacy of the GA approach. This involves genetic modification of embryos and quantitative assessment of editing approaches. These experiments are typically performed in vitro, limiting the creation of animals with sub-optimal genotypes. Further, the efficiency/efficacy of the approach informs the total number of animals that should be created to ensure project success.

- The use of well-established methods and protocols aiming to the use of the minimum numbers of mice in production of genetically altered animals. For example: establishing the CRISPR methodology in contrast to ES-cell microinjection, choosing wild type strains for embryo generation and to use as foster mothers, using the most reliable method for superovulation, optimising the numbers of embryos transferred to surrogate mice avoiding the use of surplus female mice and at the same time having less animals undergoing surgical procedures

- Purchase of wild type strains from approved suppliers in order to avoid the generation of surplus animals from in house colonies

- Collaborating with peers to refine and reduce mouse numbers used in protocols, eg through the use of genetically sterile mice as opposed to surgical vasectomisation

- Literature studies, in order to assess any known phenotypes.

- Cryopreservation of gametes and embryos to archive lines will avoid wastage from the need to maintain colonies by continuous breeding .

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal model that will be used is the mouse. Mice are widely used to gain an understanding of the complex cellular interactions that underpin both health and disease. Mice are well adapted to the laboratory setting, their small size makes it permissible to house multiple animals in a space and cost- efficient manner, and they are generally easy to handle and dose. Much of its biology and biochemistry mirrors that of human, and the mouse genome has been well characterised

The transgenic team will use highly refined and widely accepted methods for the generation of GA mice and there is adequate literature to support the standardization of protocols, methods and reagents that will be used. For example, I am exploring the possibility of replacing surgical vasectomised mice with wild type hybrids (Replacement of surgical vasectomy through the use of wild-type sterile hybrids https://rdcu.be/cc2CF).

Why can’t you use animals that are less sentient?

Because mice are the least sentient animals for the purpose of this license. The mouse genome mirrors the human genome, is genetically sequenced, it is relatively easy to manipulate the mouse genome, for example, adding or removing a gene to better understand its role in the body. In addition, many GA strains are already available and well characterised. Furthermore, they are close to human physiology and there is a wealth of literature to support the studies.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- By adapting any new techniques discovered in the transgenic field, always in line with the 3Rs principles.

- By encouraging the end users to cryopreserve their mouse lines, in the case that they are not needed in the short term.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The transgenic team will use highly refined and widely accepted methods for this type of work and there is adequate literature to support the standardisation of protocols, methods and reagents that will be used. Published guidelines for best practice will be followed, including:

Refinement and reduction in the production of genetically modified mice; Laboratory Animals Vol 37, Supp 1 July 2011. (http://la.rsmjournals.com/content/vol37/suppl_1)Assessing welfare and severity of GA mice under Directive 2010/63/EU. https://www.rsb.org.uk/images/assessing-animal-welfare-.pdf Genetically altered mice. https://www.nc3rs.org.uk/GAmice

Guiding principles on good practice for Animal Welfare and Ethical Review Bodies. October 1998. 3rd Edition - September 2015.

http://www.lasa.co.uk/PDF/AWERB_Guiding_Principles_2015_final.pd

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

By:

- Attending in all the relevant scientific meetings and conferences

- Reading papers

- Establishing a good communication with Named Persons.

- Being an ongoing member of International Society for Transgenic Technologies.

- Being an ongoing member of Hellenic Society of biomedical and laboratory Animal Science (HSBLAS member of FELASA).

- Being an ongoing member of European Federation on Animal Science (EAAP).

173. Preclinical validation of intraperitoneal delivery of chemotherapy for treatment of ovarian cancer

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Chemotherapy, Peritoneum

Animal types	Life stages
Pigs	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Using a pre-clinical, translational animal (porcine) model, this project will test and validate therapies used to treat abdominal cancer, in particular ovarian, in situ.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Ovarian cancer is the biggest gynaecological killer of women in the UK with around 7,500 women diagnosed each year. Current treatment includes extensive surgery and chemotherapy. However, only 46% of women diagnosed with ovarian cancer survive

beyond 5-years. Previous research has shown that administrating Hyperthermic Intraperitoneal Chemotherapy (HIPEC) directly into the peritoneal cavity in situ (e.g. immediately after removal of the cancerous ovaries) has a positive clinical benefit to the patient in terms of increased patient survival rate and reduced cancer recurrence. HIPEC is only currently used in the surgical theatre. A recent development in delivery of peritoneal chemotherapy has been Normothermic Intraperitoneal Chemotherapy (NIPEC), which can be used outside of the operating theatre. However, to date NIPEC has only been used sparingly, due to low patient compliance when conscious, an inability to tolerate the treatment, often due to catheter infection and toxicity, when left in situ for many weeks. Working with a UK company with experience of both HIPEC and NIPEC, a newer system has been developed that enables chemotherapy to be administered into various body cavities but with accurate flow rates, at minimal pressure and optimal temperatures. The newer system will enable patients to receive a clinically superior administration of intraperitoneal chemotherapy, by using a bespoke catheter, which is inserted into the patient during surgery and can remain in situ for up to 6 months, with limited toxicity. This project will test and validate the new catheter design in a relevant pre-clinical model, before first-in-man clinical testing in association with medical colleagues working in the NHS.

What outputs do you think you will see at the end of this project?

Technology: This project works with a UK company currently delivering world-class solutions for the multi-billion dollar and rapidly growing marketplace of hyperthermic (heated) cancer treatments. The company have developed a unique modular recirculating system enabling chemotherapy to be delivered to patients at tightly controlled temperature, pressure and flow-rate with accuracy and control significantly greater than current state-of-the-art. The platform can perfuse and optimally deliver drugs into different organs by varying the flow rate and pressures. After securing IP for the novel delivery platform and clinical procedure, then this new technology, once validated in pre-clinical models, will enable the company to push for National Institute for Clinical Excellence (NICE) approval to adopt into the NHS for the benefit of improved patient care and recovery post-surgery for abdominal cancers.

Researchers in the UK: the data generated as a result of the work in the porcine model will be published open-access in peer-reviewed journals. Therefore, the information will be disseminated amongst scientists, clinicians and technologists primarily within the surgical and oncology communities. There is considerable interest in HIPEC for treatment of abdominal, particularly late-stage ovarian, cancer. Potential new treatments with high efficacy and fewer side-effects, as we envisage this new technology to be, are always welcomed warmly by the oncology community.

International researchers: Ovarian cancer is the biggest gynaecological killer of women in the UK with around 7,500 women diagnosed each year. Globally, this equates to 239,000 women. The economic burden associated with ovarian cancer in the UK, differs considerably depending upon the stage at diagnosis; stage 1 treatment costs £5,328

compared to stage 4 costing £15,081 [IncisiveHealth Report, 2014]. With 7,500 women being diagnosed with ovarian cancer in UK alone, then the economic impact in the UK and Internationally are huge. Current treatment includes extensive surgery and chemotherapy. Despite this, only 46% of women diagnosed survive 5-years post diagnosis. Hence, success of the current project will be of interest to the many 00's of researchers around the globe with a vested interest in ovarian cancer, and also other cancers affecting the abdominal cavity or peritoneum.

Who or what will benefit from these outputs, and how?

Short-term: The platform used to deliver intraperitoneal chemotherapy, together with the new catheter that this project will test will provide the first clinical solution for late-stage ovarian cancer patients where cancer has metastasised to the peritoneum. The preclinical work described in this project is a part of a large, funded project which, after successful pre-clinical validation of a new intraperitoneal delivery system (rate of recirculation, temperature, pressure, dose of chemotherapy all to be validated) will, within 2 years, be tested directly in patients attending NHS surgical oncology units.

Longer-term: The successful output of the project will be a unique intraperitoneal chemotherapy delivery system with preliminary clinical data, as a stepping stone to a larger clinical, trial prior to full clinical adoption (within 3 years). The overall benefit being the reduction of cancer-recurrence and significantly enhancing the 5-year survival rate of ovarian cancer patients.

How will you look to maximise the outputs of this work?

The data generated as a result of this work will be published open-access in peer-reviewed journals. Therefore, the information will be disseminated amongst scientists, clinicians and technologists primarily within the surgical and oncology communities. There is considerable interest in HIPEC for treatment of abdominal, particularly late-stage ovarian, cancer. Potential new treatments with high efficacy and fewer side-effects, as we envisage this new technology to be, are always welcomed warmly by the oncology community. Internationally, ovarian cancer is the biggest gynaecological killer of women, approximately 239,000 women per year. The economic burden associated with ovarian cancer within the US alone, has been estimated to be $6.03billion in 2020. Current treatment includes extensive surgery and chemotherapy. Despite this, only 46% of women diagnosed survive 5-years post diagnosis. Hence, success of the current project will be of interest to the many 00's of researchers around the globe with a vested interest in ovarian cancer, and also other cancers affecting the abdominal cavity or peritoneum. All information will be shared through conferences, publication and technology/industry events.

Species and numbers of animals expected to be used

- Pigs: 30

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will focus entirely on using the pig as the preclinical animal model of choice. That is because the technologies we are testing and developing will be adopted by the NHS whom require that any medical device, such as a catheter, that is going to be placed into a human body cavity must first be tested on an animal model. For this project, the abdominal cavity of a young adult pig (e.g. 50-70kg, 6-8 months of age) is of similar size and volume to a human patient and therefore allows similar rates of peritoneal flow e.g. upto 2.5L/min (average being 2L/min) that will be used clinically. Initial pre-clinical testing of scaled down pilot devices has been developed using rats (in Spain). Now, with a final product ready for potential use in humans then a pig is the most translational, applicable animal model. For studies of short-duration, where the similarity between porcine and human physiology is key to achieving the primary outcome, then certain breeds of commercial pig e.g. Landrace or Tamworth are suitable due to their size when young adults.

Typically, what will be done to an animal used in your project?

In this project, typically, female pigs will be placed under general anaesthesia and a peritoneal catheter will be inserted using a minimally-invasive approach and attached to a machine set to deliver peritoneal lavage at 2L/min for 90mins (with or without dissolved chemotherapy drugs). After the final sample, pigs will be humanely killed whilst still under anaesthetic using barbiturate overdose. Blood samples may be taken before and during the procedure to test, for example, for uptake of differing chemotherapy drugs. The rate, volume and pressure of the delivered peritoneal solutions will be varied to test for the optimal strategy that can then be used clinically. The system will be tested in a staged design; initially, 1 pig on one day to check all procedures. A second pig the following day to validate procedures. Then, upon need, one or two pigs per day to achieve our objectives, “validate a catheter used to deliver intraperitoneal chemotherapy”, that is in terms of the pressure, flow-rates and temperature of the lavage fluid. It is not expected that more than n=3 pigs will be needed for this phase. A further study may then be conducted likely involving n=12 pigs with n=4/group where the conditions optimised in study 1 will be applied to determine the distribution and dissolution of chemotherapy drugs (e.g. three groups of four pigs), as measured via peritoneal lavage and blood sampling.

What are the expected impacts and/or adverse effects for the animals during your project?

The pigs in this study will not be recovered after surgery. Therefore, the process of sedation and general anaesthesia involves a moderate level of harm experienced by the

animals but all are mitigated by effective anaesthesia and non-recovery. Pigs are also prone to porcine stress syndrome when housed individually. Therefore, we will always make sure that any pig is within sight, smell and touch of another pig while conscious.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity level is non-recovery for the pigs in this study. All pigs 100% will experience this severity. Some mild (not moderate) distress may be experienced during initial sedation and induction of anaesthesia.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

We cannot first test a new medical device (catheter) designed for clinical use in Man, without first testing using an appropriate pre-clinical model. Therefore pre-clinical testing must first use an appropriate animal model. We consider the commercial pig appropriate due to size, immune similarity to human and anatomical similarity. Previously, rodent models have been used to develop the normothermic technology. This study is to validate a larger, new catheter that would allow normothermic delivery of intraperitoneal chemotherapy in a larger cavity, as occurs in pigs and humans. Since the catheter is a new medical device with a newly engineered recirculation design, allowing for normothermic perfusion at different rates of perfusion, temperature and pressure (to hyperthermic perfusion). Therefore, rodents and other less sentient animals are inappropriate to achieve our primary end-points.

Which non-animal alternatives did you consider for use in this project?

Not applicable. The peritoneal lavage system has been extensively tested by the company prior to being used in an animal model. However, the primary outcome is clinical use and therefore pre-clinical testing must use an appropriate animal model. This particular treatment system cannot be used on non- animal alternatives.

Why were they not suitable?

Not applicable. The peritoneal lavage system has been extensively tested by the company prior to being used in an animal model. However, the primary outcome is clinical use and therefore pre-clinical testing must use an appropriate animal model. Worms, flies, fish and lower sentient animals such as mice are inappropriate for various physiological reasons. Some testing has involved rats but now due to scaling up the technology for final testing then pigs are the most translationally relevant species.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

I use specific experimental design software to calculate sample size. For any pilot trial we start with n=1 for testing, and would consider n=3 pigs sufficient to demonstrate effectiveness of the catheter such as during optimisation of either 1) flow rate, 2) temperature and 3) pressure of the recirculating system. For final testing, the primary outcome will be uptake of chemotherapy via the peritoneal route and for this aspect we do not envisage needing more than n=4 pigs per study group (at three dose levels, n=12 in total). A study group may comprise different doses of drug, but using a factorial design n=4 will factor up if no effect of dose delivered in the peritoneal gavage solution. We will not be quantitatively determining chemotherapy uptake as a result of optimising pressure, temp and flow rate. Therefore, we are not powering the trial using mean, standard deviation and effect size. Rather, we are testing if the catheter works effectively over the time period used clinically (90 mins) and whether the ideal temp, pressure and flow rate do not cause catheter failure. Assuming a 1 in 20 chance of failure by chance (0.05), then with n=4 no fails there is only a 1 in 160,000 chance that the design was optimal by chance (0.5 x 0.5 x 0.5 x 0.5 = 0.00000625). This is an acceptable risk for the study.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

At all stages, the minimum number of animals is considered. For the first phase of the project - pilot testing of a new peritoneal catheter - then n=3 animals is sufficient to demonstrate effectiveness of the catheter while we vary flow rate, temperature and pressure of the recirculating system. This takes a within-animal experimental approach to reduce between-animal variability and reduce sample number required. With the primary outcome being asked, "Does the new catheter work effectively?" i.e. deliver fluid at the required flow rate, temperature and pressure in a stable and repetitive fashion, then n=3

for pilot studies and n=4 for any treatment group using a factorial design is considered satisfactory. The investigators will use experimental design software to guide and refine experimental design and sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Pigs will be sourced from an accredited breeder. All studies will be staged as pilot studies initially. For final design of testing different chemotherapy drugs then power calculations will be used using information from pre-clinical testing in rats. We will be collaborating with NHS abdominal cancer specialists e.g. those specialising in ovarian cancer, and thus any tissue required for pump-priming projects or novel testing will be provided. We do not expect to use more than n=8/pigs per study group, although for pilot testing this will be no more than n=3 pigs in a staged design. If uptake of chemotherapy drugs is highly reproducible between pigs, then we will limit pre-clinical testing of the effectiveness of the catheter to n=3-4 pigs, as the experimental outcomes are not dependent on showing statistical significance of chemotherapy drug uptake, rather effectiveness of the peritoneal catheter.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Pigs sourced from an accredited commercial supplier which are guaranteed to be of high health status and from a strain likely to be free of malignant hypothermia, will be sourced, acclimitised for 7 days and subsequently placed under general anaesthesia. They will be sedated beforehand, then intubated and maintained using inhalation anaesthesia. They will not be recovered from the procedure as no outcomes relate to post-surgical recovery. Therefore, no animal will experience any harm beyond an intramuscular injection, intubation whilst sedated. Therefore we do not envisage any animal experiencing any pain, suffering, distress or lasting harm.

Why can’t you use animals that are less sentient?

These animals will be terminally anaesthetised. Rodent models have been used to develop the technology and are largely proof-of-principle. The need for the use of pigs is because we need to establish the temperatures, pressures and flow-rates that would be used in human translational studies. For any novel medical device that is to be used

invasively in humans then it has to be tested in an animal model beforehand. The abdominal cavity of rats and/or other less sentient animals, are too small to allow for similar conditions for the use of HIPEC or NIPEC treatments abdominally.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Initial sedation via IM injection may involve mild/moderate, short-term distress for all animals. All pain management during surgical induction and maintenance will be undertaken after consultation with the NACWO, Named veterinary Surgeon and colleagues/peers whom are experts in large animal anaesthesia. Refinement in surgical technique is always a consideration such as maintaining aseptic technique, use of local anaesthesia at the site of cannulation or catheterisation of vessels for blood sampling. Anaesthetic monitoring and depth of anaesthetic to be used will be at a minimum to achieve deep anaesthesia and constantly monitored. In addition, during GA we will monitor the pedal reflex, corneal response and depth of breathing to ensure appropriate depth of anaesthesia during the procedure. All GA will involve a mechanical ventilator to control the depth of breathing and thus anaesthesia by a competent person. Peritoneal catheterisation and lavage will be conducted according to best practice as used clinically in the NHS (a clinical oncologist will be present initially to ensure appropriate catheterisation and lavage is done according to NICE guidelines).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

There are few published best practice guidance for pigs, but all pain management during surgical induction and maintenance will be undertaken after consultation with the NACWO, Named veterinary Surgeon and experts in large animal anaesthesia.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I am a member of AWERB at our University and of the University's research ethical review panel. The PPL holder is therefore in regular contact with an NC3Rs representative on the committee, particularly AWERB. At all meetings we discuss how to best stay informed and implementing best practice in our research studies. In addition, the study researchers keep up-to-date with developments in the 3Rs via publications such as the ARRIVE guidelines, or journals such as laboratory animal research or the NC3Rs website. This way the PPL holder is kept informed of any advances that may benefit the project and the welfare of the animals

174. Understanding the neuronal network involved in the regulation of glucose metabolism, feeding behaviour and energy expenditure

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Obesity, Diabetes, Blood glucose levels, Energy Expenditure, Brain regulation of metabolism

Animal types	Life stages
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand how the central nervous system senses hormones like Insulin to regulate blood sugar levels, food intake and energy expenditure (how we burn calories).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The study of the brain regulation of energy balance has the potential to improve current treatments of diabetes and obesity. Insulin is released by the pancreas and goes to the liver to decrease glucose production (sugar released by the liver) and to promote glucose storage as glycogen. However, when insulin reaches the brain, it can act at multiple levels and have important functions like regulating the nervous system that controls how much we eat, blood sugar levels, cognition and memory and can affect mood. Deficits in brain insulin signalling have been linked to several neurodegenerative diseases including Alzheimer’s and Parkinson’s disease. Indeed, with Type 2 Diabetes (T2D) older adults suffer from decreased verbal learning, abstract reasoning, and complex psychomotor tasks. Restoring insulin sensitivity in the brain could be a good strategy to counteract the negative outcomes of obesity and diabetes. In addition, it could be beneficial for people suffering from neurodegenerative disease. The aim of this study is to investigate how the brain regulates glucose metabolism, feeding behaviour and energy expenditure. We will identify which brain cells are sending signals to the liver in order to control glucose levels and to the brown fat in order to control energy expenditure. We will also study how alteration of these brain cells can have deleterious consequences for the metabolism in particular in obese and diabetic models.

What outputs do you think you will see at the end of this project?

The involvement of the brain in controlling metabolic functions is well known, however, the impact that obesity and diabetes have on brain functionalities is understudied. The long-term effect of brain insulin resistance (decreased ability to respond to insulin) can increase the chances of developing neurodegenerative diseases. Memory loss is a big problem in diabetic individuals and this is due to the brain not functioning properly. Understanding the molecular mechanism that triggers insulin resistance in the brain is the first step for developing a specific treatment that targets the brain to ameliorate metabolic functions and also to prevent long term deleterious effects. In addition, we plan to determine the specific brain cells that are involved in insulin sensing and are affected by insulin resistance, diabetes and obesity.

Our work looks at mechanisms, our major output will be in the form of peer-reviewed publications. We are also involved in public engagement activities where we make aware the public of the effects that obesity and diabetes can have on brain functions.

Who or what will benefit from these outputs, and how?

The study of the brain regulation of energy homeostasis has the potential to improve current treatments of diabetes and obesity. Insulin is released by the pancreas and goes to the liver to decrease glucose production and promote glucose storage as glycogen. However, when insulin reaches the brain, it can act at multiple levels and have important functions, for example, regulating neural circuits that control appetite, blood glucose levels, cognition and memory and can also affect mood. Deficits in brain insulin signalling have been linked to several neurodegenerative diseases including Alzheimer’s and Parkinson’s disease. Indeed, with T2D older adults suffer from decreased verbal learning, abstract

reasoning, and complex psychomotor tasks. Restoring insulin sensitivity in the brain could be a good strategy to counteract the negative outcomes of obesity and diabetes, in addition, it could be beneficial for people suffering from neurodegenerative disease.

How will you look to maximise the outputs of this work?

The major output for this research will be of interest to the scientific community, the outcomes will be published in peer-reviewed journals. We will also present our data at conferences. The focus of this research is of significant interest to the general public, most people know someone who has T2D. In order to bring this research to the attention of academics in other disciplines and to the general public, we will work with the University Communications Office to ensure timely Press Releases. In addition, I will also present the work at the local public events organized during the science week.

As obesity is increasing amongst the young population, this project could bring awareness regarding the dangerous effect that overnutrition has on the brain and the bad consequences that this could have during development. An activity focused on this topic and targeted to young generations will be extremely important. To engage with young people, I will develop an exhibition based on our work to be used at the Festivals of Science organized locally. These events are attended by many 9-19-year-old schoolchildren. Both events are run annually and draw in around 1200 people per day from the local community.

Species and numbers of animals expected to be used

- Rats: 1200

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The experimental procedures proposed require the use of 8 to 12 weeks-old Male Sprague Dawley (SD) rats. These are fully developed rats that are still growing. We measure changes in body weight, and we aim to look at body weight increase over time. This is the best system to perform metabolic studies since their glucose levels are very stable. All our previous data are based on 8 to 12 weeks old male SD rats and in addition, they have a higher than 95% rate of survival after brain surgery. The stereotactic surgery is very easy to perform in rats and, due to their anatomy, is very easy to get the right coordinates to insert the Intracranial (ICV) cannula in the dorsal vagal complex (DVC) of the brain. In addition, the rats have fast and complete recovery after surgery. We measure changes in blood glucose levels using a pancreatic euglycemic clamp, SD rats have a stable blood glucose that make our analysis reliable over time. We have vast experience on using this

model and we can make the best use of these rats with the confidence to achieve the highest level of care and rate of survival until the end of the experiment.

Typically, what will be done to an animal used in your project?

First, rats will be subjected to brain surgery in order to insert a cannula in a specific brain area. The surgery lasts 10 minutes and is done under balanced general anaesthesia. The cannula is held with a small implant made of dental cement. Once in place, the implant does not cause any distress or pain to the rats. Rats are expected to make a full recovery within 6-12 hours after surgery. We will use the brain cannula to inject inactivated viruses that will be used to express specific proteins in the brain area we are interested in. In addition, the cannula will be used to deliver specific treatments in the brain.

On the same day of brain surgery, a subgroup of rats will also receive a small incision either in the abdominal cavity or the scapular area in order to expose the liver and the brown fat respectively. This procedure is done in order to inject an inactivated virus that can be taken up by neuronal terminals in these peripheral organs and can be visualised in the brain. After the viral injection, the small incision will be sutured. The viral injection will not cause any harm, disease or pain to the rats and the cut will be small enough to not cause post-surgical discomfort. Pain-relieving drugs are also given routinely to all animals that are subjected to surgical procedures and pain score is recorded post-surgically.

Insertion of catheters in the jugular vein and artery will be done in a subgroup of rats a week after brain surgery. This is also a moderate procedure. We will use the catheters in order to infuse substances and withdraw blood for analysis at the end stage of our experiments, which involve the injection of substances and serial withdrawal of blood for glucose tolerance test (GTT). These catheters also allow us to withdrawn blood from fully conscious rats and without the need for manual handling and repeated punctures of the vein. After vascular surgery, the rats are expected to have a full recovery and the catheters do not cause discomfort.

In order to monitor the effect that our brain treatment has on vital parameters like heart rate and blood pressure, we will implant subcutaneously telemetric devices. The implant has no effect on the day-to- day life of the rats and allows continuous monitoring of important physiological parameters.

What are the expected impacts and/or adverse effects for the animals during your project?

The majority of the procedures will require recovery from anaesthesia following surgical procedures. The surgical procedures will involve the insertion of cannulas in specific areas of the brain for the purpose of infusing substances and the insertion of catheters in the blood vessels in order to infuse substances and withdrawal of blood. Brain cannulas are inserted using specialized equipment. We have extensive experience in performing such surgical procedures and do not envisage adverse effects during the post-surgical period. Post-operative pain relief is routinely given to all animals and pain response is monitored

on a score sheet. After surgery, the rats will be housed individually to avoid the dislodging of the catheters. We will use transparent cages near each other so the rats do not feel isolated. At the end of the experiments, animals will be humanely killed to allow for tissue collection. The genetically modified rats will not have an adverse phenotype. The use of the Cre recombinase expression system under a specific promoter will allow us to target only specific cell populations. This will be very important to identify the neuronal network involved in insulin sensing and resistance in the DVC. We will use PET, MRI or CT scan to monitor fat deposition ad brown fat activity in high fat diet-fed obese rats. This will help us understand how changes in brain activity can affect whole-body metabolism. These are terminal procedures performed under full anaesthesia. Finally, during imaging sessions, we will use telemetric devices implanted subcutaneously to monitor the heart rate of the animals.

Rats will be also placed in a cold environment (cold challenge) to stimulate the release of heat by the brown fat (thermogenesis). This experiment will be performed in fully anaesthetized rats at the end stage of our experimental procedure.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All the procedures we will perform are expected to be of moderate severity. We expect a full recovery after brain and vascular cannulation. We expect that both the brain implant and the vascular tubing will not cause discomfort to the rats.

Our procedures include glucose clamp and intravenous tolerance tests to measure how the rats control their blood glucose. In both cases, the rats will be fully awake and free to move in the cage. The use of vein cannulas and long tubing will allow us to infuse substances and withdraw blood without causing any distress or harm to the rats.

The brain infusion is also painless and done through a long tubing so that the rats can be kept unrestrained in their cages.

Cold challenge and bioimaging (PET, MRI and CT scans) will be done in fully anaesthetized rats so no discomfort will be caused.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Since this study focuses on understanding the neuronal network involved in the brain control of metabolic functions and the physiological impact that insulin resistance, obesity and diabetes have on brain metabolic regulation, it is not possible to substitute the use of animals with alternative systems. We will study how the brain controls sugar metabolism, food intake, body weight and energy expenditure. For these studies, there is no possibility to use an alternative system for animals.

The experiments will be performed in rats; a vast amount of background data in relation to metabolic parameters is available which make these the most appropriate species in which to perform our studies. In particular, rat glucose levels are very stable, in addition, stereotactic brain surgery is very easy to perform in rats and, due to their anatomy, it is very easy to get the right coordinates to insert the brain cannula. Our research team has extensive experience in this kind of surgery. In addition, the rodents have a fast and complete recovery after ICV surgery and also recover well after vascular surgery.

However, where in vitro work can be undertaken we will do so e.g. all the validation of the adenoviruses and lentiviruses will be performed in cells, and only viruses that show a clear effect in vitro will be then used in vivo. Whenever an analysis can be performed using cells, we will do so instead of using rodents. We have available a plethora of brain cell lines that we can use when is needed.

Which non-animal alternatives did you consider for use in this project?

No animal alternative can be used.

Why were they not suitable?

Since we need to do neuronal tracing to look at the connection between different organs and the brain. The changes in whole-body metabolism can be studied only in the whole organism.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals that will be used in this project is based on power calculations that are based on prior experiments. To determine the required number of animals we used the variance estimated from prior work as well as expected effect sizes. We will conduct our

experiments to be able to publish to the Arrive guidelines: https://www.nc3rs.org.uk/arrive-guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The number of animals will be minimized in several ways:

Tissue and plasma from the same animal will be used for different objectives where possible. This reduces the total number of animals that would otherwise be required if a single animal was used for each experiment.

Tissue for neuroanatomical procedures such as immunohistochemistry will be stored long-term for future use where possible.

The NC3RS experimental design assistant will be considered for planning: https://www.nc3rs.org.uk/experimental-design-assistant-eda

Rodents are subjected to seasonal changes; this could affect the results in terms of high variability. In order to minimise this event, we will make sure to conduct all the experiments with the appropriate controls and to minimise the environmental changes that could affect the analysis. We will randomise the animals starting from the brain surgery (the first procedure that we perform) and when possible, we will match the body weights of control and treated subjects

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

At the end of each experiment, we will harvest as many tissues as we can even if they are not useful for the moment because they might be needed in future. In this way, we will not have to repeat the experiment again.

For example, in our recent study published in Molecular Metabolism, we performed feeding studies and harvested at the end of the experiment different organs including the brown fat (BAT). We then realized that there were changes in BAT and we performed a series of analyses that led us to a completely new project for which most of the data has come from animal tissues that we already have.

We also collaborate with other groups in the university. The hearts, muscles and spinal cord of our obese and diabetic rats will be taken by different groups in the university that are interested in the effect of obesity and diabetes on heart functions, muscle activity and the spinal cord's ependymal cells functions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the

procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The aim of this study is to look at the brain regulation of glucose metabolism and feeding behaviour. We will look at the effect that the insulin resistance in the CNS has on the whole-body metabolism. The experimental procedures we propose require the use of 8 to 12 weeks old male Sprague Dawley rats.

These animals provide the best system to perform metabolic studies. In particular, their glucose levels are very stable and this makes them the perfect model to perform pancreatic clamp studies. All our preliminary data and previous studies have been performed with rats.

We have been performing brain and vascular surgeries for years now and we have been able to optimise the procedure in order to minimise the suffering of the rats and to obtain close to 100% success and recovery after surgery. Here are some examples of how we proceeded to refine our procedure:

1) At first, we were using injectable anaesthetics that remain in the system longer, delay the recovery after surgery and sometimes can cause an overdose of the rats. We now mainly use isoflurane that is well accepted by the rats and allows the rats to wake up soon after surgery, in this way we have a faster recovery and no issues with overdosing.

2) After surgery, the rodents are housed in single transparent cages in order to avoid any potential fights that could affect the surgical areas. Initially, we tried to pair housed surgically prepared animals but noticed that animals were chewing at each other's surgical area and losing their cranial implants, rendering the animal unusable. For this reason, single housing after surgery is imperative in this study. Animals are kept in visual contact with each other and are only singly housed for a maximum of 18 days

3) After vascular surgery we prepare food mushed with water and leave it in a small dish on the cage floor. This makes it easier for the rat to eat and keep hydrated right after surgery. In addition, extra bedding and nesting materials are also provided to all surgically prepared animals for cage comfort. Heated pads are also available to aid thermoregulation during recovery after anaesthesia

4) We experienced that Adenoviral injection in the dorsal vagal complex (the area of the brain we are mainly interested in) if injected on the day of surgery, is sometimes not well tolerated by the rats. This area controls the breathing and if the rats are not fully awake after surgery, they might stop breathing. We now inject the virus via intra-cranial

cannula the day after surgery, this allows the virus to be well tolerated and no side effects have been seen.

Why can’t you use animals that are less sentient?

Since we are studying how the brain controls blood glucose levels, thermogenesis and feeding behaviour, we need a fully developed animal. We are looking at how the brain regulates peripheral organs so using organoids won't be suitable for us.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All surgical procedures are performed under balanced general anaesthesia. We use inhalation anaesthesia using isoflurane which causes rapid induction and uneventful rapid recovery. In addition, full aseptic conditions are maintained for all surgical procedures and we follow LASA Guidelines for Aseptic Surgery.

Brain surgery requires the insertion of an ICV cannula in a specific brain area. The surgery lasts less than 10 minutes and is done under full anaesthesia. After surgery, the rodents are expected to have a

full recovery within 1 day. The cannula implanted has a very mild impact on the day-to-day life of the rodents and will be kept for up to 18 days.

Vascular surgery requires the insertion of catheters into the jugular vein and artery of the rodent. The surgery lasts ~30 min and is done under full anaesthesia After surgery, the rodents are expected to have a full recovery within 2 days. The implanted catheter has a very mild impact on the day-to-day life of the rodents and will be kept for up to 4 days.

All the surgical procedures will be carried out under aseptic conditions. After surgery, we will constantly monitor until animals regain full consciousness and we will give analgesic to the rodents for 3 days until we can see a full recovery. To limit suffering, animal responses will be monitored.

Viral injection in the liver will require a laparotomy of the abdominal area to expose the liver and perform the viral injection. The injection of the virus will not cause any harm and we will do the smallest incision we can in order to avoid discomfort after surgery.

Similar attention will be observed when we will do the brown fat viral injection that occurs at the level of the scapulae in the back of the rat.

The feeding protocol is very mild, we just remove the food in the morning (the rodents eat less in the daytime). The brain infusion is carried out with a long catheter that allows the

rodents to move freely in the cage to cause them minimum stress. The infusions through the vein catheter and blood withdrawal through the arterial catheter are also carried out with long tubing that allows the rodents to freely move to minimize stress.

For MRI/PET/CT imaging, motion artefacts from breathing and heartbeats can degrade the quality of imaging – meaning that the imaging may take longer to get quality data. By implanting telemetric ECG monitors, we can get the imaging on the heartbeats and make sure that images are only taken at the correct time, resulting in better quality images and shorter overall scans.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the NC3R best practice guidance. We attend webinars from Nc3R like "the best practice in experimental design": https://www.nc3rs.org.uk/events/webinar-best-practice-experimental-design.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive newsletters from the NC3R, we rely on the website to find important information regarding ways to reduce, refine and replace (https://www.nc3rs.org.uk/). We also have regular talks with NACWO and veterinarian in order to refine our procedures.

Over the years we have changed the way in which we anaesthetize the rats, the way in which we perform post-surgical care and the way in which we keep the rats after surgery in order to improve the success rate of our procedures and the wellbeing of our animals. This has also reduced the number of animals needed for our experiments.

175. Target investigation and characterization of potential medicines

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Development, manufacture or testing of the quality, effectiveness and safety of drugs, foodstuffs and feedstuffs or any other substances or products, with one of the following aims mentioned in paragraph (b)

Key words

therapy, cancer, aging, vaccine, immune-mediated

Animal types	Life stages
Mice	adult, aged
Rats	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project is to support the investigation of potential targets for treatment of diseases and perform an assessment of the possible effectiveness of potential treatments before animal safety tests and human clinical trials.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are many areas of unmet clinical need that significantly impact both lifespan and quality of life.

Many chronic diseases, often associated with an aging population, have a significant impact on individuals' ability to live independently, resulting in significant human and societal costs. Examples include rheumatoid and osteoarthritis, Alzheimer's disease, cystic fibrosis, heart disease and stroke, liver fibrosis and inflammatory bowel disease.

Despite the recent advances made in its treatment, there are still many types of cancer that are not currently curable (for example pancreatic, some breast cancers).

New targets and approaches to treating these diseases will improve quality of life of both patients and their families, as well as potentially lessening the burden on society associated with caring for patients.

What outputs do you think you will see at the end of this project?

The studies performed under this licence will contribute to the understanding of the role of previously identified molecular targets for treatment. This may be by improving confidence that medicines and other methods of altering these targets will result in a significant benefit for patients, or alternatively that they are not involved in the disease of interest so different targets should be explored.

Where there is confidence that altering a target is likely to provide patient benefit, further studies will be performed using animal models to examine the efficacy of specific potential medicines, with a view to identifying those treatments with sufficient promise to take forward into safety assessment and ultimately human clinical trials.

Where significant results are not subject to confidentiality agreements they will be communicated more widely at scientific meetings or published in peer reviewed journals.

Who or what will benefit from these outputs, and how?

In the short term clients, project teams and funding bodies will be able to make progression decisions to be able to better target their limited financial resources on to the potential treatments with the highest likelihood of clinical success.

In the medium term stopping the progression of projects with little chance of success (either due to lack of relevance of the target or the inability to develop a potential medicine that has a clear beneficial effect in animal models) will also mean that animals will not be used in safety assessment and human volunteers will not be put at risk in clinical trials.

In the long term the successful identification of validated disease-modifying targets and treatments will result in improvements in lifespan and quality of life for patients and carers.

How will you look to maximise the outputs of this work?

Where there may be broader interest in an animal modelling approach, and if the studies are not subject to confidentiality agreements, these will be published or shared at relevant conferences.

Clients and collaborating partners will be encouraged to publish all results in journals or share at relevant conferences.

Species and numbers of animals expected to be used

- Mice: 15000
- Rats: 5000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Adult mice and rats (including genetically altered animals) will be used as these have been demonstrated to provide information that enables decisions to be made on the progression of projects. Using genetically altered animals allows the investigation of human-specific targets and treatments for these targets.

These animal studies serve as a bridge between computer or cell-based experiments and human clinical trials. These studies will, in many cases, be the final studies investigating the likelihood of a potential medicine having a benefit before clinical trials are started.

Aged animals may occasionally be used when studies investigating diseases strongly associated with older patient groups a specific age group are undertaken. Such conditions include rheumatoid arthritis, anaemia and immune senescence.

Typically, what will be done to an animal used in your project?

Typically animals will be purchased from a commercial supplier (including genetically altered lines) and will then:

- be used in procedures where blood and tissues samples are taken under general anaesthesia from which the animal does not recover. This would be a small number of animals, usually genetically altered, and the tissues would be used for cell-based tests.

- be dosed with potential medicines usually by oral, subcutaneous or intravenous injection on one or more occasions to investigate the action on the target system of interest. This would usually be no more than 4 times daily for up to 7 days but in approximately 5% of studies

may be up to 3 months (for example when investigating treatments for chronic diseases).

- To understand how a potential medicine affects a disease pathway (for example inflammation), a challenge may be administered prior to dosing. This would usually be in the form of a single dose (<5% of studies may require more than one dose) of a chemical or drug known to affect the pathway of interest.

These studies may include using genetically altered animals with relevant targets systems deleted or human-specific target systems inserted.

Occasionally (<5% of studies) surgical cannulation of blood vessels may be required to make administration and/or sampling less stressful for the animal than using a non-surgical approach

The effects of the target or potential medicine will generally be assessed by looking for markers in the blood meaning samples will be taken at intervals relevant to the disease. Blood samples taken may also have the levels of potential medicine assessed to allow the amount of effect on the tissue or target to be understood.

Typically this will be no more than 4 samples a day over 7 days. For longer studies, fewer samples will be required.

Non-invasive imaging (<10% of studies) may be performed to follow the effect of the target or potential medicine. Typically this may be up to 4 times in one day, or daily over 7 days.

- be administered an experimental vaccine no more than once weekly on up to 3 occasions during 24 week period, usually subcutaneous injection. Blood samples would be taken approximately weekly to assess the degree of immune response.

What are the expected impacts and/or adverse effects for the animals during your project?

Administration of substances causes stress and pain due to handling and needle insertion. These are controlled by skilled handling and minimising the numbers of administration and sampling events.

Some genetic alterations can cause adverse effects which are very dependent on the gene. We plan to only use genetically altered animals that have Mild adverse effects.

Challenge models are expected to stimulate the immune system and/or inflammatory pathways which can produce "flu-like" effects. These effects are expected to be Mild and short lived in most cases.

Where severity is greater and treatment is possible without negatively impacting the scientific purpose of the study these effects will be reduced by use of drugs such as paracetamol.

Anaesthesia for imaging can result in heat loss and unpleasant experiences when recovering. Animals will be closely monitored, have heat supplied throughout the imaging session and only undergo the minimum number of anaesthetics required to give a satisfactory scientific output.

Surgical cannulation of blood vessels will cause pain that is controlled by the use of pain-relieving drugs: generally this lasts for 48 hours, but the animals are closely monitored in case further doses are required.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Rats and mice 80% Mild, 20% Moderate.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Many of the biology studies performed to help in the validation of targets and assessment of potential medicines now take place in computer, genomic, receptor and cell-based experiments (as well as definitively in clinical trials in the patient population). Integrated cell-based systems (the "organ on a chip" or even "human on a chip") are improving year on year and replacing animal usage.

However, even with these advances, computer and cell-based approaches still do not allow the response of the target or potential medicine to be assessed in a way that reflects the full complexity of an integrated biological organism. This project aims to provide the data from a complex, integrated organism to allow decisions to be made on whether to progress targets or potential medicines to the next stage of development.

The use of non-mammalian species is also seen as a potential replacement approach, and useful information can be obtained from these approaches: however due to the degree of differences at a genetic and molecular level these models are more suited to very early scientific investigations rather than drug discovery and development.

Which non-animal alternatives did you consider for use in this project?

Some or all of computer modelling, genomic, cell-based and non-protected animal approaches will be used prior to undertaking studies on protected animals to minimise the number of studies and impact on the animals.

Clients will be asked to provide information on the work undertaken with approaches not using protected animals and an outline of literature reviewed searching for alternative approaches prior to performing in vivo studies. It would be expected that in vitro binding, cell-based biomarker assays and where possible human tissue studies will have been undertaken.

These studies will usually include ability to affect the target, assessing the properties of potential medicines for biological availability and likelihood of reaching tissues of interest and looking for early indications of toxicity liabilities. These test with reduce the numbers of experiments performed and increase the likelihood of those completed to deliver meaningful results.

Why were they not suitable?

There are no non-animal alternatives that can currently replicate the full complexity of the mammalian body. Now and for the foreseeable future there will need to be animal experiments performed to investigate target validity and the efficacy of potential medicines as part of the process of bridging from non-animals studies to clinical trials.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have based these estimates on the number of projects we anticipate supporting, with typical studies comparing genetically altered and/or treated animals with control animals. The estimate is that we will be supporting the in vivo work for in the region of 5 client drug discovery programmes plus occasional fee for service stand alone studies at any time under the authority of this licence.

Currently these models are predominantly available in mice but a significant number of models for disease are still performed in rats. Based on published studies a typical study may use 6 animals per group and up to 8 groups, including positive and untreated control groups, and multiple compounds at different dose levels. Running fewer, larger studies will reduce the number of positive and negative control animals used, whilst providing greater confidence in the results.

We additionally anticipate providing tissues from genetically altered animals for cell-based research where these are not available from other commercial sources at a suitable quality. The estimate is for approximately 6 animals per week to be used for this purpose.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We will use pre-existing data (published and internal pilot data) to perform power calculations that ensure group size is likely to provide a robust experimental output without using excess animals. This will usually be with the support of a biostatistician, but if this is not possible power calculations will normally be performed e.g. using the NC3R's Experimental Design Assistant.

In rare cases where there is no initial data to guide power calculations the Mead resource Equation will be used to estimate sample size.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Wherever possible pilot studies will be performed to help design the decision-making studies. If studies have been performed on how the potential medicine is absorbed and removed from the body, modelling will be used to predict the doses required for these studies. Using these pilot studies to understand how the experimental measures vary will also help design the decision making studies.

In some studies (especially those with a higher risk of toxic side effects) tissue samples will be taken after the study is completed and may help prevent further studies being performed should unexpected results be found.

When providing tissues and blood an email distribution list will be used to allow sharing of tissue throughout the company for use in cell-based studies, minimising the overall number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Genetically altered animals that demonstrate no or only Mild signs will be used.

Models where a pathway of disease interest is stimulated (e.g. by bacterial cell wall fragments, other biologically active materials or compounds that stimulate a specific inflammatory response) will be chosen to investigate whether a target is significantly involved in or a potential medicine affects that pathway. These are chosen as they have fewer adverse effects than traditional animal models such as arthritis or inflammatory bowel disease. Whenever possible the models will be managed to a Mild severity.

Vaccination models will be managed with pain-relief medications to reduce any adverse effects that may occur.

Why can't you use animals that are less sentient?

Animal models will only be used at a stage in the research programme when it is essential to study the target or potential medicine in a fully-integrated organism allowing the complex interaction of many body systems to be assessed.

Prior to this some or all of computer simulation, simple receptor or cell-based studies, complex cell- based systems (e.g. organ on a chip) or simple animals (fruit flies, nematode worms) will have been performed to focus on potential medicines that have the highest chance of success.

Most studies will be investigating responses that develop over many hours or days so the use of terminal anaesthesia is not appropriate. In addition, anaesthetic agents are likely to interfere with many of the targets of interest.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

On site, working proactively with the AWERB, NVS and NACWO, there is a culture of constant improvement to animal care, control of adverse effects, performance of procedures and study design.

Scoring systems are used to identify early intervention and end points in studies, pain-relieving drugs are used when there is concern an animal is suffering, and monitoring is performed as often as required, including throughout the night.

Further refinement to technical procedures and housing are implemented when they are shown to be beneficial for the animals and will not interfere greatly with performing the studies.

When an established model is identified from the scientific literature and proposed for use under this licence, a small number of pilot studies will be performed to ensure the model delivers the expected changes in the pathway or tissue of interest. These pilot studies will also be used to determine the controls that may be able to be used to reduce the severity experienced by the animals and the study duration.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

In addition to Home Office and EU guidance documents, relevant best practice guidance will be sourced from the NC3Rs (e.g. ARRIVE Guidelines, blood sampling, experimental design), NORECOPA (e.g. PREPARE guidelines), LASA (e.g. blood sampling, drug administration, aseptic surgery) and model-specific publications.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

On a monthly basis the PPL Holder receives and reviews automated literature alerts on animal models and journals relevant to the project licence.

When a new disease area is proposed, a thorough literature review is performed to review the potential animal models to determine the most scientifically relevant whilst causing the least harm, and a new automated alert generated.

In addition to conference attendance, webinars and discussion groups are participated in by the PPL Holder and scientific staff.

176. In vivo assessment of novel injectable hydrogel biomaterials for spinal disc repair

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Intervertebral disc, sheep, biomaterial

Animal types	Life stages
Sheep	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to examine whether a novel injectable self assembling peptide gel in combination with glycosaminoglycan (PEP-GAG), delivered by a customised small bore needle system can effectively repair intervertebral disc damage in a naturally occurring sheep model.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Lower back pain is the main cause of disability worldwide and affects many people. Lower back disease causes soreness and stiffness in the lower back and there is no specific cure, with many people suffering from lifelong discomfort and loss of function. There are a number of causes of lower back pain but one of the commonest is damage to the intervertebral discs of the spine. These discs act as shock absorbers between the bones of the spine. When the discs become damaged they lose their shape and shrink, which leads to pain and discomfort in the back.

At the moment, treatment of lower back pain relies primarily on physiotherapy and pain killers and there is an unmet clinical need for a direct treatment of disc damage. One strategy for treating disc damage is to inject the shrunken discs with a substance to increase the size of the disc and restore its function.

However, currently, products that are suitable for disc enlargement are very thick and need to be injected into the disc through large needles, which themselves cause further damage.

What outputs do you think you will see at the end of this project?

The intended output of this study is to test a new type of biomaterial, PEP-GAG, to treat disc degeneration, a significant cause of lower back pain and to develop a novel small bore needle system for delivery of the biomaterials. This output will be communicated to the scientific and lay audience by publications in peer-reviewed journals and presentations at conferences.

Who or what will benefit from these outputs, and how?

The data derived from this project will be used by scientific researchers, clinicians and veterinarians. Regardless of the success, or otherwise, of this biomaterial, researchers in the field of biomaterials and tissue engineering, especially those in the area of disc augmentation will benefit from the outputs as these studies will inform their research by identifying whether the biomaterial used is optimised for disc augmentation and, if it proves not to be, what information can be derived from this. This project has developed a system for injection into the disc by a small bore needle which will have applications in treating disc disease and other clinical situations. If this project does identify a successful product for disc augmentation, clinicians who are treating patients with disc degeneration and the patients themselves will benefit, as we will have developed a new product to treat disc degeneration and lower back pain. However the products would require clinical trial approval before use in patients.

Short-term benefits

In the short term the results of our experiments will benefit researchers working in the area of biomaterials development, tissue engineering and inter-vertebral disc disease. Our experiments will provide information on the compatibility of PEP-GAG hydrogels in the the intervertebral disc, the safety and efficacy of these preparations. In addition, the

development of a small bore dual injection needle, that allows the delivery of low viscous solutions, will provide equipment that can be used for variety of clinical uses in addition to disc injection. Both the design of the needles and the full delivery system have been completed. A device that can be used in both the sheep study and subsequent clinical trials has been designed.

Medium- and long-term benefits

Will be to clinicians and their patients as we are developing new therapies and a new delivery system for administering these therapies to treat intervertebral disc degeneration and lower back pain. If successful, these biomaterials and their delivery device, will be taken forward to Phase 0/1 clinical trials in human patients before being licensed to treat human patients. As stated above, lower back pain is the leading cause of disability worldwide. In this application we aim to show efficacy of new biomaterials, delivered via a novel small bore injection needle. If successful, this therapy could be used worldwide in a large number of patients as it is will not damage the disc on injection, as other disc augmentation materials are known to do and will provide physical expansion of the disc. This will reduce disc size loss in clinical patients and reduce back pain, leading to reduced disability, less days off work and a reduction in burden to the NHS and other healthcare providers.

How will you look to maximise the outputs of this work?

We will be collaborating with different research groups to develop our technology further. We will publish the results of this study and present at conferences regardless of the outcome, because the results will benefit groups working on other disc treatments who have an interest in the model as well as the therapy itself.

Species and numbers of animals expected to be used

- Sheep: 18

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

It is not possible to achieve the aim of this project without using animals as the only authentic test of a product designed to treat intervertebral disc degeneration is to place it into damaged discs and quantify its effect.

Sheep have been used to test implant devices and in the preclinical investigation of cellular therapies to support spinal fusion and disc reconstitution. Ovine discs, like human discs, undergo chondroid metaplasia with skeletal maturation because of the loss if their notochordal cell remnants. Additionally, the ovine disc is closer in size and cellular

phenotype to the human intervertebral disc than small animal models, important characteristics given the nutritional limitations associated with the central regions of the disc. Furthermore, despite its quadrupedal conformation the sheep spine has been shown to exhibit significant biomechanical similarities to the human spine.

Typically, what will be done to an animal used in your project?

Following non regulated behavioural monitoring to establish baseline behaviour and activity, the sheep receiving either the product or sham injection will then be anaesthetised and have an MRI or CT scan to identify and quantify the reduction in disc height and width and the presence of signifiers of degeneration such as osteophytes around the disc margins. Within a set period of time after the scan, for example 4 weeks, these same animals will be anaesthetised and have up to three lumbar discs injected under X-Ray guidance (maximum injection volume 0.5ml per disc).

We are working with naturally occurring disc degeneration and so there will be individual variation between animals and discs. To mitigate against the effects of this variation we will be imaging discs before and after treatment and comparing results of each disc to itself (size and appearance), so that each disc acts as its own control.

The animals will be housed indoors in small pens in groups of between three and six sheep for a week post-surgery and monitored clinically for any signs of pain following injection and then allowed to roam free in a field/barn for the remainder of the study during which further Fitbit and clinical monitoring will be undertaken.

At the end of this time, the animals will be euthanised and when dead will be imaged under MRI or CT before the spinal sections are extracted. From each animal, pairs of treated and untreated control discs will be evaluated using histology, immunohistochemistry and biomechanical testing.

What are the expected impacts and/or adverse effects for the animals during your project?

The overall severity of this procedure is moderate, which all animals receiving injections will reach transiently due to injections into their intervertebral discs.

We are not expecting animals to show clinical signs of increased pain or distress following injections. The injections are performed under general anaesthesia and so will not be experienced consciously by the animal. The animal may show mild signs associated with recovery from general anaesthetic, such as appearing slightly ataxic and sedated, but this is transitory and our experience is that sheep are usually standing within 15 minutes of the end of the general anaesthetic. Analgesia will be given for 72 hours post-operatively, unless an animal is assessed to require additional pain relief.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The overall severity of this procedure is moderate, which all animals in the experimental group or sham injection group will reach transiently as they will all have injections into their intervertebral discs.

Control sheep will not experience a spinal injection, but may have standing x-rays carried out.

What will happen to animals at the end of this project?

- Killed
- Rehomed
- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

It is not possible to achieve the aim of this project without using animals, as the only authentic test of a product designed to treat intervertebral disc degeneration is to place it into damaged discs and quantify its effect.

All products will have been thoroughly tested in vitro for biocompatibility and cytotoxicity before delivery into the sheep. In addition sub-cut testing was carried out in mice, and no adverse inflammatory response was detected.

Which non-animal alternatives did you consider for use in this project?

In vitro work was carried out to ensure the cytotoxicity and biocompatibility of the materials, but there is no in vitro model of spinal disc degeneration.

Why were they not suitable?

It is not possible to achieve the aim of this project without using animals as the only authentic test of a product designed to treat intervertebral disc degeneration is to place it into damaged discs and quantify its effect. In addition, testing the material in a large animal model is a regulatory requirement, so we therefore are a stage closer in translation to clinic.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will always seek to use the minimum number of animals necessary to achieve the objectives of the project whilst maintaining statistical validity of results. We have based the numbers of sheep required on similar studies in sheep and goats that have been published in the literature and are using 6 animals per experimental group. The experimental design is a simple one - we will compare the efficacy of treating disc degeneration with a novel biomaterial compared to a sham injection of phosphate buffered saline (PBS). In our research we will randomise our experiments to avoid bias, for example we randomise which animals get which treatment i.e. biomaterial or sham and in what order they undergo procedures. We will analyse our data in a blinded fashion and use coding systems to anonymise data, thereby reducing unconscious bias. In some instances we may ask our collaborators to randomly allocate animals to experimental groups and to retain the information as to which group and animal belongs until the final results are collected. We believe that these methods contribute to the robustness of our data interpretation. We are committed to working to the PREPARE guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The NC3R Experimental Design Assistant was used to create a study plan. This allowed us to critique the plan and run a power calculation. Values from published literature (change in disc height) was used in the EDA as this model has not yet been carried out by our group. Setting the power of the experiment at 80%, $p<0.05$ and a standard deviation of 2.5 we require a minimum of 6 animals per group.

We will also maximise the data obtained from each sheep e.g. activity data, post-mortem biomechanical, histological and immunohistochemistry data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Initially cadavers (collected at the end of another experiment) will be used to ensure that the injection techniques are of a high standard and in the correct location. A pilot study of three animals will then be used to ensure that the 3 disc injections do not compromise animal welfare but also to ensure that the material does not cause inflammation within the spine and that the sheep have a smooth recovery. The pilot sheep will remain within the study for the full 12 weeks, and their data will be included in the study.

At the end of the study any unwanted tissue may be harvested by other groups for further research.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We have chosen the sheep as our model for a number of reasons. Firstly, the sheep exhibits naturally occurring disc degeneration and, by using these naturally affected animals, we avoid the need for painful interventions to induce disc degeneration. Secondly the sheep has an intervertebral disc of similar structure and anatomy to human intervertebral discs, making the translation of results from sheep to human more direct and, thirdly, the sheep has a sufficiently large intervertebral disc to allow injection of the hydrogel through a needle of similar size to that which would be used in man. The only other alternative, suitable species with naturally occurring disc degeneration is the goat, however, our group has considerable expertise in anaesthesia, surgery and management of sheep and considered that using sheep was within our sphere of knowledge when goats would not be. Thirdly, by using sheep exhibiting disc degeneration, we also avoid the uncertainty involved in artificial induction, which can produce variable levels of degeneration and may not mimic the naturally occurring pathophysiology.

We will be conducting a small stepwise pilot study of three sheep to ensure that the 3 disc injections/sheep proposed do not compromise animal welfare and that this injection regime is suitable to take forward to the main study. We will minimise animal suffering by ensuring that animals receive anti-inflammatory medication during and after spinal injections. We will monitor animal suffering by assessing their activity and behaviour via video and remote telemetry (using ‘Fitbits’).

The ‘Fitbits’ allow us to continuously record the ‘play’, ‘rest’ and ‘activity’ of the sheep before and during the experiment. This allows us to identify early indicators of reduced welfare in the animals and to intervene accordingly. If reduced activity or play and increased rest is noted, the NVS will be asked to examine the animals to identify the cause of the reduced mobility.

Why can’t you use animals that are less sentient?

It is not possible to achieve the aim of this project without using animals as the only authentic test of a product designed to treat intervertebral disc degeneration is to place it into damaged discs and quantify its effect.

The sheep are aged animals and will be assessed before spinal injection to confirm that there have naturally occurring degenerative changes in their lumbar spine.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will be conducting a small stepwise pilot study to ensure that the 3 disc injections/sheep proposed do not compromise animal welfare and that this injection regime is suitable to take forward to the main study. We will minimise animal suffering by ensuring that animals receive anti-inflammatory medication during and after spinal injections. We will monitor animal suffering by assessing their activity and behaviour via video and remote telemetry (using ‘Fitbits’). The ‘Fitbits’ allow us to continuously record the ‘play’, ‘rest’ and ‘activity’ of the sheep before and during the experiment. This allows us to identify early indicators of reduced welfare in the animals and to intervene accordingly. If reduced activity or play and increased rest is noted, the NVS will be asked to examine the animals to identify the cause of the reduced mobility.

All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

The sheep will receive analgesia (e.g. fentanyl patches), as advised by the NVS, pre-operatively and post-operatively to allow for a continuous supply of analgesia for an appropriate period. In addition the sheep will receive local anaesthetic in the injection/incision site. To reduce the risk of infection the sheep will receive antibiotics pre-surgery and post-surgery. Non-steroidal anti-inflammatories will be used for up to 72 hours after injection, unless already being administered to ameliorate the effects of aging.

We will grade the spinal pain and in addition there are three clinical score sheets to guide us when monitoring the animals, one covers the mobility, food intake and water consumption, fever, demeanour and respiratory rate and is routinely used for all animals for a minimum of 3 days post-operatively.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All surgery will be carried out aseptically in dedicated facilities, according to the LASA guiding principles for preparing for and undertaking aseptic surgery.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

During the study we will be in contact with the NACWO and the NVS in addition to monitoring any updates from the NC3Rs website.

177. Vascular calcification in kidney dysfunction

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

vascular calcification, chronic kidney disease, calcium

Animal types	Life stages
Mice	pregnant, adult, juvenile, neonate, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To understand the mechanisms that lead to the hardening of blood vessels in patients with chronic kidney disease (CKD).

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Patients with CKD, diabetes and atherosclerosis have an increased risk of bone-like calcium deposits forming in their blood vessels. This process, known as vascular calcification, causes the blood vessels to become stiffer. Consequently they cannot stretch as easily as normal blood vessels, resulting in an increase in blood pressure which in turn places more stress on the heart. As a result, patients are more likely to die prematurely.

Currently there is no effective treatment for this condition. Therefore there is a clinical need to understand the underlying disease process in more detail so that potential drug targets can be identified.

What outputs do you think you will see at the end of this project?

The project has two arms, approaching the theme of vascular calcification from two different angles.

In the first part of the study we will use a genetically altered mouse strain that we have shown is more prone to developing vascular calcification than normal mice. One aim of the current study is to establish whether a drug that blocks the pathway activated as a result of the genetic modification is effective in inhibiting vascular calcification. As patients with CKD are known to show similarly increased activity in this pathway, by demonstrating the effectiveness of the drug our study would provide strong evidence for a new therapy.

In the second part of the study we will use a different genetically altered mouse strain to explore the relationship between calcium and phosphate in the development of vascular calcification. We have shown that the molecular sensor which detects the amount of calcium in the blood, and regulates the secretion of the hormone that controls the release of calcium from bones, can also detect phosphate. We have altered the phosphate sensing part of the molecule and predict that this will protect mice with CKD from developing vascular calcification. If that is the case this would point to towards a highly specific drug target that could not only reduce vascular calcification but may also be useful in the treatment of the bone and mineral disorder commonly found in patients with CKD.

Who or what will benefit from these outputs, and how?

In the short term, the project will help us to better understand the processes that lead to the formation of mineral deposits in blood vessels. This knowledge will help other researchers who are working in this field and more widely those who are seeking to understand blood vessel function and the control of calcium balance in the body.

In the longer term, the project may lead to the development of new treatments for patients suffering from vascular calcification. Both arms of the project could lead to the development of new drugs or the repurposing of existing drugs for the treatment of vascular calcification.

How will you look to maximise the outputs of this work?

The genetically altered mice strains that we have generated are likely to be of interest to other researchers, so we would be open to collaboration and the sharing of materials to maximise the impact of our work.

We already collaborate with other research groups, both at our own institution and elsewhere, so we envisage that the outputs of the current work will feed into further

refinement of techniques and research partnerships. For example, we have collaborated with colleagues with expertise in imaging to develop a method which allows us to make two separate sets of measurements in the same sample. Previously, two tissue samples from two separate mice would have been necessary to achieve the same outcome.

We will make data sets, including those with negative outcomes, available to other researchers via the institution's data repository.

Species and numbers of animals expected to be used

- Mice: 1400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use mice that have been genetically modified in one of two ways. One strain lacks the ability to make a target molecule of interest, making them more prone to developing vascular calcification than normal mice. In this way we can study the impact of the loss of this molecule on the calcification process and its related impacts on other parts of the body, such as the kidneys. The other strain of mouse has received a modification to the molecular sensor which detects calcium in the blood. We predict that a certain part of this sensor will also detect phosphate, and as such that it plays a role in the bone and mineral disorder that accompanies CKD in patients. We have changed the structure of this molecular sensor so that it no longer responds to phosphate, and in doing so we anticipate that these mice will be less prone to developing vascular calcification than normal mice.

We are using these mice because they have highly specific changes in the function of the molecules of interest. This means that we can study the impact of this altered function in the whole animal, thereby allowing us to model the complex disease process that occurs in patients with CKD and vascular calcification.

In order to generate mice for use in experiments, we will have to maintain breeding colonies of both strains of genetically modified mice. We will only use adult mice in the subsequent experiments, in order to model the form of vascular calcification that occurs in adult humans.

Typically, what will be done to an animal used in your project?

Mice will undergo a two-step surgical procedure to remove 5/6th of their kidney mass in order to induce CKD.

In the first part of this process, 2/3rd of one kidney are removed under anaesthesia. This involves making a small incision into the abdomen just below the ribs to access one of the

kidneys. It does not matter which kidney is chosen, as they are identical in structure and function; however we typically start with the left kidney as it is easier to access. The upper and lower parts of the kidney are removed, tissue glue is applied to stop any bleeding and then the wound is closed and the mouse is allowed to recover. Analgesics are administered to prevent the mouse from experiencing pain.

7-14 days later a similar procedure is undertaken, again under anaesthesia. This time the whole of the second kidney is removed. The wound is closed and analgesics are administered to prevent the mouse from experiencing pain.

At least 7 days after the second surgical procedure has been completed, the mouse's diet is switched from normal chow to one that contains a higher level of phosphate. This is done to speed up the development of vascular calcification. The mouse will be maintained on this high phosphate diet for up to 16 weeks, but typically 8 weeks, before being killed and tissues collected for further analysis.

In some experiments, a small device will be implanted under the skin on the animal's back, between its shoulder blades, in order to deliver a drug. These tiny pumps are able to deliver a drug at a constant rate for up to 4 weeks: where a longer period of drug delivery is required the pump may have to be removed under anaesthesia and a replacement implanted. This would only happen on one occasion extending the total period of drug delivery to 8 weeks.

In other experiments a drug may be administered orally by inserting a tube briefly down the mouse's throat and into its stomach: this is called gavage. This approach will be used rather than dissolving the drug in drinking water and allowing the animal to self-dose as it is important to ensure that each mouse receives the correct dose. It is also helpful when drugs are not soluble in water, or are unstable in aqueous solutions over several hours, or are very expensive which prohibits making up large volumes. This method of drug delivery would be used once daily for up to 4 weeks.

Small blood samples will be taken every 7-14 days for up to 8 weeks by pricking the mouse's tail with a sharp needle. A small drop of blood will be collected in order to assess markers of kidney function.

What are the expected impacts and/or adverse effects for the animals during your project?

The genetic modifications do not cause any overt effects, so neither strain of genetically altered mouse is expected to suffer from any adverse effects prior to entering an experimental procedure.

Surgical removal of 5/6 of the kidney may cause acute kidney injury, a condition in which the animal's remaining kidney mass is not able to function adequately to remove waste products from the blood. The risk of acute kidney injury is minimised by performing the

surgical procedure to remove the kidney tissue in two stages with at least 7 days recovery in between each step.

After the first surgical procedure the animal is unlikely to suffer any adverse effects and post-operative pain can be prevented by administering analgesics.

If acute kidney injury does occur, it is likely to do so in the period 10-24 hours after the second surgical step. The signs of acute kidney injury include lethargy and a lack of interest in food or water, fur standing on end and a pinched facial expression. Administration of fluids and analgesics by injection may help to alleviate these symptoms; however if there is no improvement the mouse will be killed to prevent further suffering.

The surgical procedure is designed to induce CKD in the mouse; however the animal will not experience the clinical consequences of this condition as the experiment will end before this stage is reached.

Once the mouse has recovered from surgery it will be fed a diet containing elevated levels of phosphate: this is designed to speed up the formation of calcium deposits in the blood vessels. The diet itself is palatable to mice, so they will eat it readily; however some animals can lose weight rapidly over 24-48 hours. Placing the animals temporarily in a warming cabinet can minimise this loss of weight, which will be detected by regular weighing.

Feeding a high phosphate diet in combination with removal of 5/6th of the kidney tissue will result in vascular calcification. Although this has adverse consequences for patients, which is the rationale for studying the condition in this project, the mice will be killed and tissue collected for further analysis long before they experience the clinical consequences of this condition.

The drugs that we plan to administer are not reported to cause any adverse effects at the intended doses and duration of treatment.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severity level associated with the breeding and maintenance of the genetically altered mice is mild.

Some of those mice will then undergo the surgical procedure to induce CKD and subsequent feeding of a high phosphate diet, which has an expected severity limit of moderate.

Therefore approximately 70% of mice will experience mild levels of suffering and 30% experience moderate levels of suffering.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

CKD leads to complex changes in physiological function, both within the diseased kidney and in the body as a whole. Many of these changes are not understood fully and so cannot be reproduced using isolated cells or computer modelling. However it is precisely these systemic changes that result in vascular calcification. Consequently, it is necessary to study the changes that occur in blood vessels in the intact animal in order to understand what is happening as CKD progresses. This leaves us with no viable alternative to the use of animals for the proposed project.

Which non-animal alternatives did you consider for use in this project?

We have used isolated cells to perform many of the experiments that underpin this project and we will continue to do so where appropriate.

Why were they not suitable?

Isolated cells are useful for providing proof of principle and for testing drugs or understanding at the molecular level how signalling processes occur. Where they are less useful is in trying to understand the more complex interactions that occur in the organ or the whole body, especially in disease states. CKD is a complex disease which can alter the way that the body regulates calcium, ultimately leading to vascular calcification. This process cannot be modelled accurately using cell culture.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The majority of animals used in this project will be required to generate and maintain breeding colonies of the two strains of genetically altered mice. Hence these numbers are based on the prior experience of both our group and that of numerous other researchers at our establishment and elsewhere.

For the remaining animals, we are able to base our estimates of the number of mice that will be required on our previous experience with the main experimental model.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have designed our experiments using the NC3R's Experimental Design Assistant so that we are able to gain the most possible information from each individual animal.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In maintaining our genetically altered mice colonies we will employ efficient breeding strategies to ensure that the number of excess animals is kept to a minimum. Colony size will be reviewed regularly to ensure that breeding matches the anticipated demand for experimental animals.

We have designed the experimental plan in such a way that there are a number of points at which a decision is made whether to continue with a particular objective(s) or not. Thus if an experimental outcome indicates that there is no value in continuing, that aspect of the work will cease thereby preventing the unnecessary use of animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The purpose of the animal model is to induce vascular calcification in mice in a way that mimics disease progression in humans with CKD. To do so, first we need to induce CKD. This can be achieved in a number of ways in the mouse, including exposure to radiation, obstructing the outflow of urine from one of the kidneys and administration of a number of drugs. However none of these methods is suitable for our purpose as they produce pathologies that differ from the type of disease typically seen in patients with vascular calcification.

Therefore, we will employ a surgical procedure to remove 5/6th of the kidney mass as the means to induce CKD. This involves removing kidney tissue in two stages and allowing the animals to recover between procedures. Thereafter, mice begin to develop CKD over a period of several months. As in humans, there are no overt clinical signs or pain

associated with disease progression until the final stages. However, mice will not experience pain or distress due to CKD as the experiment will finish before they have reached this point.

The second part of the process involves feeding the mice a diet containing higher than normal levels of phosphate. We know from a pilot study conducted in earlier experiments how much phosphate the mice will tolerate (1.5%) and how long we need to feed the diet in order to induce the formation of calcium deposits in their blood vessels (8 weeks), so we can have confidence that this model will work in our hands while at the same time causing the least distress to the animals. Vascular calcification associated with CKD will lead to an increase in the animals' blood pressure, but as in humans, there are no overt signs that hypertension causes pain or distress.

Why can't you use animals that are less sentient?

We are not aware of any non-mammalian animal models of vascular calcification that mimic the pathology seen in humans as they age. There is a zebrafish model of generalized arterial calcification of infancy (GACI); however as its name implies, this is a disease that manifests either before birth or early in childhood as it is a rare genetic condition.

All published studies in this area, including our own, have either used isolated cells or rodents. The former are a valuable tool with which to study molecular pathways in specific types of cells. The latter are most frequently used in vivo because of the need to model a complex, multifaceted disease process that involves the kidneys, blood vessels and hormones that regulate calcium.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

During the course of earlier work authorised by a previous licence (P217A25EF) we have optimised the dietary conditions necessary to produce the calcification of blood vessels, minimising the time required to reach the scientific endpoint of the experiment while causing the least distress to the animals.

Furthermore, we have refined our surgical procedure and post-operative monitoring regimen to ensure that any animal at risk of acute renal injury can be identified and killed humanely to prevent unnecessary suffering.

We will continue to review our procedures on an ongoing basis and make refinements where ever possible to minimise the welfare costs incurred.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

All procedures will be undertaken in accordance with institutional standard operating procedures (SOPs) and guidelines.

The approach to surgical procedures will be further informed by the Laboratory Animal Science Association's (LASA) Guiding Principles for Preparing for and Undertaking Aseptic Surgery (https://www.lasa.co.uk/PDF/LASA_Guiding_Principles_Aseptic_Surgery_2010.2.pdf).

Blood sampling will conform to the LASA Good Practice Guidelines for Collection of Blood Samples (http://www.verutech.com/pdf/lasa_blood_sampling.pdf).

When publishing the outcome of our work we will adhere to the ARRIVE guidelines (https://arriveguidelines.org/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We seek to stay informed of 3Rs advances through innovations published in the literature, discussions with colleagues at other institutions, regular updates provided by named individuals at our own institution and the local NC3Rs Regional Programme Manager and through subscription to the NC3Rs newsletter.

178. Environmental stresses in forages affecting ruminant digestive physiology

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

greenhouse gases, climate change, feed efficiency, ruminants, feed security

Animal types	Life stages
Cattle	adult
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Future climate scenarios are predicted to affect forages grown for ruminant production. Changes in hydration, temperature and carbon dioxide levels are potential stress factors for forage growth and production. Examination of ruminant digestive efficiency as affected by consuming stressed forages requires examination in regards subsequent greenhouse gas production by ruminants and the ability to increase feed efficiency to support the increase in global demand for meat and milk due to population growth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

It is predicted that global temperatures will rise by 2 degrees Celsius by 2050.
Current forage varieties have been developed under recent ambient levels of carbon dioxide and traditional weather patterns. Moving forwards, it is important to try and account

for changes in the growth efficiency of forages plants and the subsequent effect these have on the nutritional physiology and performance of ruminants. It has previously been shown within the host institution that applying environmental stresses relating to potential future climate scenarios (associated with global warming), such as varying levels of water, temperature or carbon dioxide affects post-harvest plant metabolism and rumen metabolism in vitro. However, these previous experiments have been limited and require further extensive investigation using animal models. Ruminants have the ability to utilise poor quality fibrous forage material and convert it into human edible meat and milk. Examining the effects of future climate scenarios along with the examination of nutritional efficiency will allow for plant breeding targets to be defined and inform decisions moving forwards. Production of stress tolerable forages and improving ruminant nutritional physiology may help delay the rise in global temperature.

What outputs do you think you will see at the end of this project?

This project will address how environmental stress in forage grasses and the plants subsequent response to ingestive stress affects animal production. The outputs will contribute to the understanding of the potential impact of future farming and refine models for the rumen system. It will aid in the understanding of forage feed composition and its utilisation and gain knowledge of plant traits (beneficial and adverse) when used as a forage feed. The outcomes will also relate directly to further the knowledge for animal production and environmental outputs as well as obtaining information for forage breeding for the future.

Who or what will benefit from these outputs, and how?

There will be a range of academic and non academic beneficiaries including; Microbiologists with new information about microbial community development, Plant biologists providing more information on stress responses in plants with implications for crops as well as animal forage, Environmental scientists, to understand more about climate predictors and Ruminant scientists, to gain greater understanding of the metabolism of forage feeds and utilisation within the animal in terms of animal production and potential environmental pollutants. Plant breeders will also gain knowledge of particular plant traits when considering breeding for productive animal forage feed and also Farmers will gain knowledge on beneficial forage types for the future to maximise pasture and animal performance.

How will you look to maximise the outputs of this work?

We will engage with industry practitioners and representatives by attending industry-facing shows and co-operating with the efforts of both local and national farming outreach programs. We will ensure translation of research to product via links with an international grass seed company. The improved understanding of effects of climate change on forage digestion towards the reduction of greenhouse gas emissions and thus meeting emission targets set for livestock will also maximise outputs by informing policy makers.

Species and numbers of animals expected to be used

- Cattle: 58
- Sheep: 102

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mature sheep, that have finished growing may be used as a model to evaluate the effect of environmentally stressed forages on rumen metabolism, apparent whole-tract digestibility, enteric greenhouse gas emissions and rumen microbial community analysis without interference caused by growth, reproduction or lactation. Results obtained using mature sheep can then be used to refine the design of diets for production animals such as growing lambs or lactating dairy cows where performance data can also be recorded, in order to evaluate environmental and economic benefit.

Typically, what will be done to an animal used in your project?

Animals utilised for feeding and metabolism studies may be individually housed and restrained if appropriate for the safety of both operator and animal. The maximum period of time the animals will be restrained in dairy cows will be four periods of 28 days followed by a rest period of at least 56 days.

Sheep will be housed individually in pens with an appropriate floor size, based on their body weight, for the monitoring of individual feed intake for a maximum duration of 112 days. Sheep will be transferred into metabolism crates, where movement is limited for a maximum period of 10 days within a 21 day period.

What are the expected impacts and/or adverse effects for the animals during your project?

Mild transient (1 to 2 hours post procedure) pain is to be expected for blood sampling or oesphageal rumen sampling

Abnormal behaviour may be observed when animals are individually isolated from being able to directly interact with another animal. Animals will always be able to see other animals. From previous experience individual isolation has negligible/transient effect on animal behaviour with feed intakes being normalised within 24 hours.

Weight loss is a potential impact due to stress associated with individual facilities. This will be mediated wherever possible, but if not the animal will be removed from the experiment and observed by a veterinary professional.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All other animals will only experience a mild degree of pain or suffering.

What will happen to animals at the end of this project?

Kept alive
Used in other projects
Rehomed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Ruminant livestock have a fermentative fore stomach commonly referred to as the rumen. The rumen is the site of fermentation of fibrous plant material by an interaction with an extensive microbial community. Digestion of fibre and protein from forages within the rumen and the rest of the digestive tract can meet the nutrition requirements of the animals, which in turn can produce meat or milk for human consumption.

Which non-animal alternatives did you consider for use in this project?

There are no laboratory assays available for monitoring rumen fermentation or apparent whole-tract digestibility which do not involve the use of an animal donor.

Why were they not suitable?

The rumen represents a part of the entire gastrointestinal tract and can not elude to animal performance or health.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of cattle represents two intensively monitored experiments containing 9 animals per run and a larger production study of 2 treatments containing 20 animals per treatment.

The number of sheep represents a cohort of 6 sheep to be used in intensive monitoring experiments and the use of commercial animals in 2 separate studies examining 4 treatments and utilising 12 animals per treatment.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

This is based on the minimum number of animals required to obtain valid and robust results without having to repeat the experiment unnecessarily with excess animals. There is local expertise and knowledge based upon previous experimentation in order to justify the choice of animal numbers within an experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

In order to reduce the numbers of animals used within this project pilot in vitro screening studies will be conducted under another authorised project to assess the effect of stressed forages on the rumen in both batch and continuous culture.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Cattle and sheep will be utilised within this project. All experiments relate to better understanding of digestive physiology in response to forages that have been produced under different climate stress scenarios. Previous experience and expertise within the research establishment will allow for the best animal experience possible. All methods chosen are animal and size appropriate and are those most suitable to causing the least amount of pain and distress to the animals.

Why can’t you use animals that are less sentient?

This project requires the use of live ruminant animals in order to complete the project targets. There is no other less sentient option available and terminal euthanasia would not allow for measurement of the desired parameters.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Procedures will initially be based upon best practise and refined through experience and discussion with other establishments where similar procedures have previously been undertaken.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Procedures relating to ruminant livestock are typically reported in appropriate animal science journals. Where new procedures are to be undertaken the authors will be contacted and advice sought in order to pre-empt any problems and mitigate animal suffering.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The information on the 3 R's will be regularly reviewed throughout the projects duration to ensure that any changes that need to be implemented during the project can be achieved. Communication between technical animal care staff, project supervisors and the animal care team will be maintained throughout the project.

179. Models for experimental therapeutics of childhood cancers

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Cancer, Immunotherapy, Paediatrics

Animal types	Life stages
Mice	adult, pregnant, embryo, neonate, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overarching aim is to facilitate improvements in the clinical management of children with cancer by modelling the disease, its detection and its therapies in mouse models that closely resemble the human counterpart.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cancer is the most common cause of death in children over one year of age. Those who are cured achieve this often through administration of very intensive and toxic therapies.

Use of mice with intact tumour structures and immune systems allows refinement of current clinical practice and especially the development of immunotherapies that can avoid the toxicity of conventional approaches.

What outputs do you think you will see at the end of this project?

The expectation is that by the end of the project there will be increased understanding of how drugs (chemicals, antibodies, engineered cells) can work together in combination to eliminate childhood cancers. Moreover, we aim to demonstrate how injectable agents can allow detection of childhood cancers using imaging methods, including during surgery. The net result should be the opening of clinical trials in children to evaluate the new therapeutics and imaging findings.

Who or what will benefit from these outputs, and how?

During the five years that the project is active, the anticipation is that between one and four clinical trials will open in paediatric oncology as a direct result of preclinical evaluations performed in this project.

Moreover, published work emerging from the project should contribute indirectly to rationale and design of clinical trials for childhood cancers both nationally and globally.

How will you look to maximise the outputs of this work?

The greatest impact on the clinical oncology field will be achieved through use of reproducible models that as closely as possible reflect the human disease counterpart. The reagents generated in this project will be made available to the broad community as well as our existing collaborators. New knowledge will be published including where possible robust experimental approaches that show lack of efficacy of a combination treatment.

Species and numbers of animals expected to be used

- Mice: 6050

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

All experiments are being performed on experimental mice. Mice are an optimal species for investigations of cancer immunotherapy for the following reasons:

They have a well characterised and understood immune system with a high degree of homology with the human counterpart.

There are already in existence numerous transgenic models of both immunodeficiency and cancer predisposition that are ideal for our studies on the interaction between cancer and the immune system

Typically, what will be done to an animal used in your project?

Mice will be engrafted with tumour cells to form models of solid or liquid human cancer. The purpose of these models is threefold: firstly to gain increased scientific understanding of the basic biology of tumour growth and in particular the interaction between the host tissues and cells with the growing tumour: secondly to use the cancer-bearing animals as subjects for evaluation of anti-cancer agents and interventions in therapeutic trials; thirdly to evaluate the role of cancer imaging to direct or monitor treatments.

A number of different approaches are used to generate the cancer-bearing animals including intravenous or intra-brain injection of tumour cells and, in some cases, genetic modification that promotes tumour growth. Typically the therapy interventions being evaluated will be a combination of immunotherapies or chemotherapies given by mouth or intravenous injection, irradiation of tumour and, in limited numbers of trials, surgical removal of tumour.

What are the expected impacts and/or adverse effects for the animals during your project?

Tumour growth in mice may induce local or systemic side effects. Tumours can impact on important organs or tissues through pressure effects as they grow. A range of side effects are possible depending on the position of the tumours. For example abdominal tumours might impact on the intestine, brain tumours might create local pain or decrease brain functioning. The side effects induced by tumour growth are therefore somewhat broad and will require careful monitoring both of tumour size and general wellbeing (activity, weight, feeding and grimace score for example). Tumours typically develop within 10 days of injection of tumour cells and cause local pain if allowed to grow to a lump greater than 1cm diameter. This can manifest as weight loss, ruffled fur and decrease activity. Administered therapies may alleviate suffering in some mice or worsen it in others if there are toxic side effects.

Duration of clinical trials of anti-cancer therapies is typically 3-5 weeks during which time mice are monitored for tumour size, weight and behaviour. Humane endpoints may limit the duration of trial in mice receiving no benefit from the interventions. In a small proportion of experiments, surgery or radiotherapy to tumour bearing animals may be incorporated to better represent treatments applicable to human subjects; here the total tumour and treatment burden is further enhanced by the increased number of interventions.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All work will be performed in experimental mice. Amongst protocols there is a mixture of maximum severities of mild, moderate and severe. Severe symptoms are possible because of the sometimes unexpected side effects or speed of growth. However any mice with severe side effects is likely to be very short lived since humane endpoints will be immediately applied.

Estimate of proportions are as follows:

- Mild: 30%
- Moderate 65%
- Severe 1-5%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The project is focussed on experimental therapeutics for childhood cancer that is rooted in clinical translatability. A major development in oncology is immunotherapy but many existing models of childhood cancer fail to reflect that developmental origin of childhood cancers which results in their relative lack of detection by cells of the immune system. Optimal evaluation of therapeutics interventions that evaluate immunotherapy in combination requires the solid tumour model in the context of a natural host immune system, including the system of blood vessels . This can only be achieved using an animal model.

Which non-animal alternatives did you consider for use in this project?

All therapeutics trials agents are first evaluated using cell line and organoid models to ensure proof of concept activity.

Why were they not suitable?

Cell line and three dimensional tumour models growth in tissue culture are not sufficient for evaluation of translatable therapeutics combinations since they lack three dimensional structure, vasculature and host immune response to tumour.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Total numbers of animals per protocol through the life of the project have been estimated based on the likely number of experiments to be performed over the course of the project, which in turn could be increased from current level of activity based on success rates of ongoing grant applications. However the estimates are based on the assumption that the number of funded projects will remain at the current level during the course of the license. The principle for individual experiments is that they should be definitive; ie statistical tests will be performed to determine that experiments include sufficient numbers of animals per experimental group to achieve statistical significance so experiments do not require to be repeated. Frequently small pilot experiments are needed to determine likely required group size to achieve this aim.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The main factors determining total mouse numbers for this project is the number of experimental control groups and number of mice per group. The most effective way to reduce total numbers is to power experiments correctly with sufficient numbers of animals to reduce the need for exact experimental replicates. Correct determination of control groups is dependent on correct definition of a research question and consideration that an effect must be attributable to the intervention of interest. To determine numbers of mice per group, formal power calculations must be performed for all experiments using tools such as NC3R's experimental design assistant. For the purposes of this project applications many different research questions will be evaluated and effect sizes are likely to vary. Pragmatically therefore average group sizes of 7 mice per group have been used along size an average of 6 groups per experiment which is a typical number for therapeutics intervention trials in oncology.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Efficient breeding will be used to minimise numbers; a good example from current work is the TH- MYCN-ALK transgenic strain in 129/ScJ background; wild type littermates from these breedings are used in trials not requiring transgenic background. Similarly for

breeding, careful record keeping ensures optimal generation of mice with potentially harmful phenotypes. Avoidance of breeding of old mice also reduces total mouse usage and potential for harm.

Pilot studies are the norm for determining likely effect size and, if there is no effect, in preventing a futile experiment being performed.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All the work described in the license application are based on experimental mice. For the majority of the work, mice used carry no harmful mutations. All experiments make use of cancer-bearing mice in order to evaluate anti-cancer therapeutics interventions. Experiments on cancer-bearing mice are relatively shortlived with humane endpoints in place so the duration of suffering of any individual animal is weeks or days in duration rather than months and humane endpoints can be applied before burden become moderate or severe. All mice are culled at the end of therapeutics trial interventions or when humane endpoints are reached, whichever occurs first.

Why can't you use animals that are less sentient?

Meaningful evaluation of anti-cancer treatments need to reflect the scheduling of agents that can be achieved in patients. Duration of treatment and evaluation must be sufficiently long to monitor effective cancer regression/remissions. By its nature this entails observation of tumour growth in mice engaged in normal activity over a period of weeks. The interventions used in this study require an intact immune system reflective of the human counterpart and growth of a solid vascularised tumour. There are no less sentient animals available which are suitable for solid tumour growth, administration of multiple therapeutic agents, and in the context of a mammalian immune system.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

All experimental work is carried out in our institutions's biological services facilities where mice are monitored daily by the trained technicians for well being as a safeguard to welfare, in addition to the requirement for PIL holders operating under the proposed license to monitor experimental mice undergoing interventions regularly in partnership with

the core animal technicians. An additional refinement for protocol 6 and 7 in which mice undergo surgery is peri and post operative pain relief which will be defined on protocol by protocol basis in discussion with named veterinary surgeon.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The following documents and guidelines will form the basis of best practice. All experimental planning is performed following approval of experimental protocol and all deviations or new procedures are discussed with the named vet.

NC3Rs ARRIVE guidelines https://www.nc3rs.org.uk/the-3rs

http://www.3rs-reduction.co.uk/assets/applets/Festing_Altman.pdf Guidelines for the welfare and use of animals in cancer research

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Resources available include the 3Rs webpages at (https://www.nc3rs.org.uk/the-3rs) where there is a link to 3Rs resources. At our institution there are many opportunities to discuss new protocol refinements with named vet, regularly on site and available for meetings and informal discussion, and/or via the ASRU Regulatory Advice Team, both of whom are.

180. Understanding and developing new treatments for neurological diseases

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Brain, Disease, Models, Therapy

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant
Rats	neonate, juvenile, adult, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to improve our understanding of the causes of progressive conditions that affect the brain and to devise effective therapies for these devastating diseases. These conditions have been selected through discussions with clinicians who have no effective therapy to offer their patients and also through the availability of a suitable animal model. Some of these conditions are rare while others are more common, some are caused by genetic mutations while others are an injury but it is the lack of available therapies that brings them together in this project.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Diseases that affect the brain are a major cause of disability and death in populations around the world. Some of these diseases are rare and aggressive and cause death in children e.g. Niemann-Pick type C disease and infantile neuroaxonal dystrophy. Others are more common and affect the elderly e.g.

Parkinson's disease. Due to the complexity of the brain, these diseases are difficult to treat and many cause either life-long disability and/or premature death. The outcome for the patient is devastating. For the families, carers and healthcare system it is a huge burden. The socio-economic impact of this is significant. What these conditions have in common is that they remain poorly understood and there is no cure. This project is important because it aims to increase our knowledge of these conditions by studying animal models of these diseases. This information will then be used to design and evaluate new medicines that may have the potential to provide life-saving and life-changing benefits to patients.

What outputs do you think you will see at the end of this project?

The outputs from this project will come in a number of forms.

1. We will generate new information on the biology of the brain diseases that we are studying and on the effectiveness of the medicines and therapies that we are evaluating.

2. We will publish our findings from this project in reputable and peer-reviewed journals so that the information and knowledge is shared widely.

3. We will file patents on new medicines and therapies that we develop and this is important in making them viable products that will benefit patients.

4. We will generate therapeutic products from this project that will benefit society and those suffering from these devastating brain diseases.

Who or what will benefit from these outputs, and how?

There are a number of individuals or groups that will benefit from these outputs.

1. Scientists - Publications that may arise in the short-, medium or long-term stages of the project will benefit the broader scientific community that are studying these diseases, or conditions similar to them. It will enhance their knowledge base and provide new avenues of understanding, research ideas and research direction.

2. Clinicians - Publications and data that may arise in the short-, medium or long-term stages of the project will benefit clinical professionals looking after the patients with these diseases. Published information on new findings relevant to these diseases can help inform on better care, diet, physiotherapy or ways to monitor the progression of these conditions. The development and publication of data that is supportive of a new drug will facilitate early dialogue and communications between the scientific and clinical community that are integral components of the drug development process.

3. Patient and Family Organisations - The patients and their families/carers will benefit from all new information at any stage of this project. This will help them to make important decisions on their lifestyle, care and any new medicines that may be approaching clinical trials in which they may be able to participate in.

4. Industry - The involvement of industry is an important component in developing drugs for brain diseases. Industry partnerships with academics to facilitate or accelerate the process are hugely beneficial and publications and patent filings will help to form these collaborations. The engagement with industry can also take place at any stage of the project.

How will you look to maximise the outputs of this work?

There are a number of ways that we will maximise the outputs of this work.

1. Publication of data and findings in a range of high impact peer reviewed journals that are both field specific (e.g. Journal of Neuroscience) and have a broad readership (e.g. Nature Medicine). This will ensure that our data and findings are disseminated broadly across the scientific and clinical community.

2. The data generated will be used to form new national/international collaborations. This will provide access to new technologies, analytical methods and ideas which will accelerate our objective of understanding the neurological basis of the diseases we study and the generation of new medicines to treat them.

3. Presentations of data as oral or poster communications at national/international conferences. These will include both specialised meetings e.g. targeting neuroscientists or neurologists (e.g. the annual Society for Neuroscience Meeting), or lay audiences as part of public engagement activities e.g. patient and family meetings or public engagement days (e.g. the annual British Society of Gene and Cell Therapy).

4. Responsible and professional dissemination of publications and outputs via social media platforms. This ensures very broad dissemination of outputs across society from lay followers, to students to experts in the field in the most expedient manner.

Species and numbers of animals expected to be used

- Mice: 7500

- Rats: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To achieve the aims of this project, the use of animal models of disease are required. Cell culture models do not have the complexity to fully evaluate what is happening in humans and are not sufficient for regulators to approve a drug for clinical use. These are mouse and rat models of neurological disease. There are a number of reasons why these specific animals have been selected:

The majority of animal models of the neurological conditions that are available for us to study and evaluate new medicines in have been made in mice and rats.

The complexity of the mouse and rat brain in terms of the structure and organisation is sufficient to broadly mimic that of the human brain.

The relatively short breeding times and lifespan of mice and rats means that we can complete studies of disease progression and the effectiveness of medicines we are testing in a feasible time frame.

The tests that we conduct to assess the way that neurological disease progresses and the effectiveness of medicines that prevent this are well established and understood in mouse and rat models. We have spent more than 15 years investing in and developing tests of movement, strength and the ability to walk in a manner that is least stressful for the animals.

Our overarching aim of this project is to develop new medicines that will help treat children and adults with devastating neurological conditions. This requires interactions, advice and ultimately permission from national and international regulators e.g. The UK Medicines and Healthcare Product Regulatory Agency (MHRA) or the European Medicines Agency (EMA). The regulators have approved drugs based on data in mice and rats to progress to clinical trials and the patients who need them most.

These mice and rats will also be used at different life stages ranging from prenatal to new born to adult. This is because the diseases that we are studying are paediatric and affect children before they are born (e.g. Type II Gaucher Disease), shortly after birth (e.g. Hypoxic-Ischemic Encephalopathy) or are adult conditions (e.g. Parkinson's Disease). The use of mice and rats at these different stages reflects the age of onset seen in patients.

Typically, what will be done to an animal used in your project?

The experiences that an animal will go through in the project can be divided into two main categories.

- Studying neurological disease in rodent models - Mice or rats that model a particular genetic disease will be bred in such a way that offspring inherit the defective genes from their biological parents and develop the disease, in the same way that this happens in humans (e.g. Niemann- Pick type C Disease or Gaucher Disease). Another way is to surgically or chemically induce cells in the brain to degenerate and therefore show disease symptoms e.g. a model of hypoxic- ischemic brain injury is induced surgically in mice by ligating the carotid artery that feeds blood to the brain and temporarily reducing the availability of oxygen. Or Parkinson's disease is induced in mice or rats by injecting a specific chemical into the brain that depletes the specific cells that are also degenerating in the patients. Animal models that show these symptoms will be studied to assess how the condition progresses through its lifetime and what the effects are i.e. the natural history of the disease. This can be assessed through a number of means: i) an experienced user observes the animals and measures changes that are visible compared to normal 'control' animals that don't have the disease, ii) weigh the mice to see if they are losing weight, iii) conduct behavioural assessments in the mice to see if they have any deficits in the way they move, strength, co-ordination and memory, iv) biological samples in the form of blood or urine may be collected from animals to see if there are any abnormalities (as in human patients) or 'biomarkers' of the disease and v) the animals may be culled at certain time points to study how and which organs of the body have been affected.

- Once we understand the natural history of the disease in the animal models as described above, we would then use them to test the effectiveness of medicines that we are assessing. This would involve adding the medicine to food or water or injecting them directly into the animal e.g. under the skin, into the bloodstream or directly into the brain. Some medicines require to only be given once (e.g. gene therapy) while others would require to be given repeatedly throughout life, similar to human patients on certain medication. In the case of longer treatments, the ideal scenario would be to have the drug in food or water but in some cases the medicine may require to be injected weekly or less frequently.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts of this projects are as follows:

- Pain - this can range from very transient and localised pain following a single injection of a medicine into a vein to more involved surgery where direct access to the brain is required e.g. to induce a model or deliver a medicine. In the latter case, local and general anaesthetics

are utilised for surgery followed by a course of pain relief and careful observation as the animal recovers.

- Abnormal behaviour - As in human patients, the animal models of neurological disease will have progressive behavioural abnormalities such as reduced mobility, co-ordination, strength and memory function.
- Weight loss - This is observed as the symptoms of neurological disease progress and can be a consequence of losing the ability to feed and drink.
- Weight gain - Food consumption is controlled by specific areas of the brain. We will be studying mouse models of obesity and diabetes and assessing drugs that modulate signals in specific parts of the brain to reduce food intake.
- All of the above are monitored using an agreed scoring system and the humane endpoints to ensure limits on these.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

This severity limit of this project is 'moderate' and the humane endpoints and scoring sheets reflect this. Approximately 90% of animals used in this project will be mice and 10% will be rats.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The brain is the most complex organ of the body and is made up of many different cell types. These different cells interact with each other in a very complex way. The brains of rodents such as mice and rats are broadly sufficiently complex to mimic that of human beings. This, taken in combination with the fact that models of neurological diseases or protocols to surgically or chemically induce them already exist in these species, makes them appropriate for studying neurological diseases and also the effectiveness of new therapies. Furthermore, national and international regulators that assess the case for

advancing a new therapy into humans require animal data as part of the assessment criteria. This is especially important in developing new cutting edge therapies that may be used for the first time in humans e.g. gene therapies or novel peptide therapies. Therefore, animal use is of critical importance in achieving the aims of the project which is to further understand these neurological diseases and to develop new treatments for them.

Which non-animal alternatives did you consider for use in this project?

The only non-animal alternatives would be to use brain cells that can be grown in a dish within a lab or post-mortem brain tissue

Why were they not suitable?

Growing brain cells in a dish does not mimic the true complexity of the brain and the myriad of interactions that occur. There is essentially no brain anatomy and so does not help in understanding which of the many regions of the brain are affected and when this starts. Furthermore, it is difficult to capture the many different types of brain cells into a dish. Cell models are also of limited use in trying to assess how well a drug distributes within a complex organ such as the brain (this is called biodistribution). Cell models also don't tell us how long a drug is retained in the brain and how long they are active for (this is called pharmacokinetics and pharmacodynamics).

Post-mortem brain tissue is limited in terms of how much is available for study and this is important for some of the very rare diseases that are being studied in this project e.g. Infantile Neuroaxonal Dystrophy for which there is no post-mortem tissue available. Furthermore, not all areas of the brain will have tissue available. New therapies cannot be tested using post-mortem tissue and important assessments of biodistribution and pharmacokinetics and dynamics cannot be conducted.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals we will use in this project is based on a number of factors: i) What we have learned about the models from previous projects, ii) our experience with statistics and using experimental design software that allow us to predict the number of animals required for each study, iii) taking guidance from the principles of the National Centre of Replacement, Refinement and Reduction (NC3Rs) and ARRIVE guidelines, iv) this project is part of an ongoing large funded programme of work and the totals take into account the number of experiments likely to be conducted over the time frame of this project.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3Rs Experimental Design Assistant to ensure that our animal work produces reliable and reproducible results. Through taking into account from the beginning criteria such as statistical analysis, randomisation and blinding of researchers and calculations that help to estimate the required number of animals, we reduce usage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

All the therapies we use in animals are first tested on cells in a dish to ensure that they are pure and active. This reduces sources of error and unnecessary animal usage.

Since we also use animals that are at a foetal or newborn stage, wherever possible we use specific strains of mice that have very good maternal instincts and look after their pups well. This increases survival and reduces numbers used.

Some of the models used in this project are induced through alternative surgical or chemical means rather than genetic models and does not require numerous rounds of breeding. This significantly reduces animal usage.

We also archive all tissues that are generated from our experiments at -20, -80 or -150 degrees Celsius. This ensures that unnecessary repetition of experiments is avoided and we can also share these with researchers all over the world without them having to establish their own animal colonies.

We freeze embryos or sperm for all of our mouse lines so that we do not need to breed mice unnecessarily.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The animal models used in this project mimic the human forms of neurological damage and disease. These include more common conditions (Parkinson's Disease and Hypoxic Ischemic Encephalopathy) and much rarer conditions (Gaucher Disease, Batten Disease, Infantile Neuroaxonal Dystrophy, Dopamine Transporter Deficiency Syndrome,

Manganese Transporter Deficiency, Fatty Acid Hydroxylase Deficiency Associated Neurodegeneration, Niemann-Pick Type C Disease). In most cases, these are the only animal models of the disease available. We have over 15 years of experience of working with animals models of neurological diseases and providing the highest levels of welfare.

Why can't you use animals that are less sentient?

Because we are testing new therapies that will be used for downstream clinical use, we need to use animal models that have the required levels of brain complexity and physiology. Mice and rats fulfil this criteria and are accepted by regulators such as the FDA, EMA or MHRA that need this data from these models to assess effectiveness and safety.

Gene therapy is a therapeutic modality that we are researching and assessing and the genetic sequences used should be tested in an animal model that have a genome sequence as similar to human beings as possible. Mice and rats also fulfil this criterion as their gene sequences (particularly the ones we are investigating) are very similar to that of humans.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We continue to minimise harm to animals via various means:

We have invested in buying state of the art behavioural analysis equipment that minimises stress to the animals through handling and maximises data acquisition and analysis e.g. we have a Catwalk XT gait analysis system and the ANY-maze software. We also now film our behavioural analysis so that we can review this later and in slow motion rather have to prolong analysis and stress to the animals.

We have worked with the veterinary and animal care technicians to optimise our post-operative care and pain management.

We have implemented robust monitoring score sheets that separately cater for neonatal and adult animals which monitor animal welfare and have been successfully used.

We have adopted best practice with respect to housing e.g. the use of environmental enrichment and of cylinders to pick up mice and reduce stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The experimental design resources available through the NC3Rs Resource Hub will be used. This provides access to a wealth of guidelines e.g. ARRIVE and information on the design and analysis of experiments at all stages.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in the 3Rs and their implementation into this project will be ensured through regular contact with the NC3Rs, the Veterinarian and biological services unit staff. We shall use the NC3Rs Resource Hub to keep up with best practice. All staff working on this project will subscribe to the NC3Rs newsletter and will access NC3R E-learning modules and webinars and those organised by the biological services unit.

181. Alternative pre-mrna splicing control and its contribution to tissue injury and pain

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

pain, nerve damage, arthritis

Animal types	Life stages
Mice	juvenile, adult
Rats	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This project aims to look at how an RNA editing process - called pre-mRNA splicing or alternative splicing - contributes to inflammation, tissue damage and pain.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

There are many different forms of a protein known as vascular endothelial growth factor (VEGF). VEGFs with only slight differences in their structure can have completely effects

in the body. For example, one causes pain and another blocks pain but they are 96% the same in structure. When people have painful osteoarthritis there is more of the VEGF that causes pain in their joints than in normal joints.

The type of VEGF that's made is controlled by another protein called SRPK1 by changing a process called splicing. SRPK1 might also control splicing in other proteins that are important in pain and joint damage in arthritis. We don't yet know what these other possible SRPK1targets are.

This work is important for the following reasons:

1. Lots of molecules that are known to be important in pain are spliced. We don't know very much about how that works, or how important splicing is in causing pain caused by damage to joints or nerves.

2. We are making new blockers of proteins like SRPK1 that control splicing. One of these is already in a clinical trial to treat damage to the retina in the eye. If we are to develop more new treatments, we must understand more about proteins like SRPK1 and how they affect pain.

3. We know that VEGF is spliced by SRPK1 but we don't what else is. Nerves, blood vessels, and other cells work together to sense pain to protect us, and splicing might change how all these cells function. It's important to understand what sort of splicing happens in different cells and tissues and how that might change, and contribute to pain.

4. Because splicing can change if tissues are damaged, it might mean that it is also part of the body's attempts to repair the damage. Our work will also give us more information on whether controlling splicing can help stop damage in diseases like diabetic nerve damage or arthritis.

What outputs do you think you will see at the end of this project?

1. The generation of new essential knowledge on neuronal and vascular mechanisms contributing to pain, and the changes associated with both acute and chronic pain. This is most likely to be in the form of publications or patents.

2. The application and exchange of knowledge to drive both future mechanistic research and drug discovery strategies.

3. The work we are doing in this area may influence strategies for novel drug design in industry (medicinal chemistry) in addition to identifying possible new targets for drug discovery.

4. As this work involves development of new compounds that may become treatments for pain, new analgesic therapy may be an outcome.

Who or what will benefit from these outputs, and how?

1. The chief beneficiaries of new knowledge generation will be the basic science, clinical science and veterinary science research communities. Our findings and publications will inform other work, in both academic and industrial research communities.

2. The pharmaceutical industry may benefit from this work through access to novel chemistry or information that leads to the re-purposing or reformulation of existing drugs for additional uses.

3. We have established a company through which we will develop potential drugs, and therefore the company will benefit from this work. Partners in the company such as large pharmaceutical companies and investors may also benefit.

4. Our work ultimately aims to benefit people and animals with chronic pain, so the medical and veterinary fields and patients may also benefit from this work.

How will you look to maximise the outputs of this work?

We collaborate widely and have a strong collaborative partnership with a medicinal chemist. This results in maximisation of the impact of his work in designing new molecules, and our work in drug discovery.

We publish and present work through multiple routes, researchers working on the project are encouraged to engage with academic and lay audiences in the dissemination of findings.

We have established a company through which we will develop potential drugs for clinical use.

Species and numbers of animals expected to be used

- Mice: 330
- Rats: 60

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This project will look at the ways in which specific changes in which proteins are expressed in which tissues contribute to both nervous system changes in response to pain, and tissue injury, and repair, and how we might be able to reverse such changes. As

such we use rodent models of nerve injury (neuropathy). We use adult and juvenile rodents as the conditions that we want to understand - nerve damage from diabetes and chemotherapy, and arthritis, predominantly affect juveniles and adults.

Additionally we know that early life painful experiences in humans and rodents affect later life experiences, and so study in adults cannot fully inform how juveniles perceive or develop pain in later life.

Typically, what will be done to an animal used in your project?

We will use rodent models of nerve injury that include mild surgical damage to one superficial nerve, or systemic treatment with low dose chemotherapy agents, or models of arthritis of one joint, and monitor their behaviour, including measurement of any changes in pain that they may experience. In some experiments animals will be treated with compounds with the potential to reduce pain and protect against injury/promote repair. These experiments may last between 4 and 14 weeks, depending on the aim. In these experiments, a model of nerve injury or arthritis is induced, under general anaesthesia where necessary, and following recovery, depending on the aim, some animals will be treated with analgesic/cell protective agents for up to 12 weeks.

What are the expected impacts and/or adverse effects for the animals during your project?

These experiments aim to identify mechanisms of tissue repair and pain, and as such the animals do experience mild - moderate pain resulting from nerve injury or arthritis. All models used are the most refined that still result in the necessary nervous system changes that we aim to target. The animals will experience changes in walking, after surgical nerve injury or arthritis they may guard or have reduced weight bearing on the affected hind limb for up to 3 days. After this animals usually return to using all four limbs with slightly less weight borne on the affected paw for the duration of the experiment. When chemotherapy models are used, animals do not show any change in their walking. The animals only very rarely experience any weight loss in any of the models.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The maximum severity of the procedures is deemed to be moderate, based on >30 years experience with the models. It is estimated, based on experimental design, that up to 75% of rodents might experience moderate severity, with the remaining animals experience mild severity or be below threshold for naive control animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Physiologically no cell functions on its own. This project focuses on tissue damage, inflammation and pain, which are complex pathophysiological processes involving multiple cell types, systems and interactions that cannot yet be effectively modeled using non-animal alternatives.

Sensory neurones are long cells that connect peripheral tissues in for example limbs, with the spinal cord and brain. If these neurones are removed from the body, their targets and the central nervous system, they are, by definition, damaged as they are cut and disassociated from their peripheral and central connections, which initiates a damage response. Neuronal damage in conditions such as diabetes can affect the long neuronal processes, the spinal cord neurons and blood vessels, and the brain. Using isolated cells or cell lines in vitro cannot tell us how damage in different parts of a neurone, or the nervous system, result in pain. Similarly the joint is a very complex structure where different cells (cartilage cells, bone, synovial cells lining the joint, blood vessels) that are co-dependent on each other for full function. Cell lines not only do not contain all the functional proteins found in, for example, sensory neurons in the body, they can also behave very differently. Some cells, such as neurones and cartilage cells do not normally continue to grow and divide but cell lines do, so there are fundamental differences just in basic behaviour. Using cell lines representing different joint tissue components, such as cartilage cells, cannot provide the same information as the study of cartilage in a joint where there is also movement and load bearing. The use of cell lines can therefore inform, but not replace study of specific cells and cellular interactions in an intact physiological system.

Neurons not only communicate information to the central nervous system, they also contribute to the severity of inflammation and healing responses. Removal from their normal tissue surrounding significantly alters these functions. The complex interactions between neurones, and the cells which surround them in the both CNS and in peripheral tissues are what changes neuronal function, inflammation and pain in a whole animal or person. These complex relationships cannot yet be replicated in vitro, or mathematically in silico.

Use of genetically modified animals, such as those with a gene knockout is required, because although knockout of a specific gene product in a cell tells us how that changes that cell's function, it cannot tell us how this then changes a system in which that cell type is fundamental. This can only be studied in whole animals.

As our aims are to not only study neurons, cartilage, synovium and vessels but also the interactions with their local environment and between cell types to fully understand the consequences and potential benefits of control of alternative pre-mRNA splicing. Ultimately these therefore need to be studied in animals.

Which non-animal alternatives did you consider for use in this project?

We have evaluated several immortalised cell lines that have similarities to sensory neurons, and we use these for initial screening experiments. They are however, only useful non-animal tools to study protection of neurones from damage, or ways in which damaged neurons can be stimulated to recover from existing damage as a first step in understanding how this could be achieved.

We have developed in vitro model of inflammation using human synoviocytes, primary endothelial cells or endothelial cell lines and fluorescently labelled immortalised monocytes. This assay enables us to screen novel inhibitors for effects on inflammatory cell and migration prior to any experiments involving animals. We also use chondrocyte cell lines to progress work and define mechanisms in relation to cartilage damage and repair in arthritis.

Why were they not suitable?

As stated above, cell based models need to effectively replicate the physiological or pathological processes we aim to study or treat, in the conditions under which they occur - pain and inflammation resulting from damage to peripheral tissues, directly to nerves, or to joints. None of the available neuronal cell lines fully replicate the primary neuronal functions that we study, namely the change in function that makes neurones more sensitive and more likely to fire electrical signals, that then lead to the sensation of pain, nor do they provide the capability to assess the interactions with multiple different cell types as found in vivo, as co-culture techniques cannot yet replicate these. These cell lines are therefore not fit for the purposes of this project.

None of the available in vitro cell based approaches can effectively model the complexity of inflammatory pain, or the pain associated with degenerative joint disease or peripheral nerve damage, they can only give indications of potential effects on the isolated cells which need to be validated in primary cells and in animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Mice

We have planned experiments for the next 2 years as follows:

Year 1. Determination of the effects of inhibitors of at least 2 kinases in male and female mice treated with chemotherapy agent vincristine, including behavioural and post-hoc tissue outcomes. For our usual experimental design with previously observed effects, this equates to 4 x 24 mice. This will be adjusted should effect sizes with novel inhibitors differ from those previously seen. Prior to these experiments we will need to determine tolerance and dosing for 2 such compounds - we cannot estimate sample sizes for these experiments until they are started so have estimated a maximum of 20 animals (both sexes) for each. Thus in year 1 we estimate use of ~150 mice.

Year 2. We have an ongoing drug discovery programme with new compounds being produced at pace and being screened in vitro. We plan on assessing at least two novel compounds in year 2 as above, so animal number estimates would be as in year 1, or slightly higher depending on experimental outcomes.

Rats

Year 1. Determination of the local (intra-articular) and systemic distribution of at least 3 novel splicing kinase inhibitor chemotypes following systemic and/or intra-articular injection (knee joint) under maintained terminal anaesthesia. This is required before any use of these novel chemotypes in an animal model to enable dosing ranging, possible use of any carrier/formulation, and choice of route of delivery. As with mice, it is not possible to estimate sample sizes yet. Animal number in year 1 is estimated to be 30 rats (5 per chemotype, both sexes), as rats are only needed when intra-articular delivery is to be used (joint size), or use of any specific model that is only available or characterised in the rat.

Year 2. We have an ongoing drug discovery programme with new compounds being produced at pace and being screened in vitro. We plan on assessing at least two novel compounds in year 2 as above, so animal number estimates would be as in year 1, or slightly higher depending on experimental outcomes and rate of movement into models.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have recently introduced a new chemotherapy model to the lab and ran a pilot study to determine

a) translatibility of the model to this environment and b) effect size of the model on the primary outcomes. The numbers planned for the first 2 years of this project are primarily based on use of this model. We use various tools, including the NC3Rs experimental design assistant and other statistical support, including consultatation with a statistician who is often a co-applicant and advisor for funding applications, to enable us to estimate numbers of animals as closely as possible.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

1. We use current technological advances, such as in data capture software, that increase ability to collect data from multiple recordings in the same animal, and experimental designs that include repeated measurements in the same animals (where and if possible), to ensure that each experiment generates the maximum amount of data from each animal.

2. We use statistical modelling in order to determine the sample sizes needed for experiments before beginning, and in all our applications for funding to support this work. Numbers of animals and experimental designs are thus rigorously peer reviewed prior to any experiments. A priori calculations are based on either our previous data in the same models, or on published work from others working in similar areas, and are used to determine numbers needed.

3. We use historical or literature controls, such as previous sham control groups in which we know (such as vehicles or sham interventions have no observable effects) for example for comparison with experiments using novel pain killers, to ensure that we do not expose additional control animals to painful conditions unnecessarily (such as injections).

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use models of peripheral nerve injury in mice, primarily surgical traumatic, and chemotherapy- induced nerve damage. Surgical models may involve a single sensory nerve (e.g. the saphenous nerve) or branches of the sciatic nerve. These models are as refined as possible by inducing damage to only some of the nerve fibres in the nerve and maintaining some nerve supply to the paw, while mimicking trauma such as might occur in accidental injury. Crush injuries or section / ligation injuries allow regeneration to occur or prevent it respectively, enabling investigation of strategies to promote regeneration, or to treat pain in circumstances when regeneration is not possible. The models that we use have been extensively refined (often by us) over the years to minimise the degree of damage and pain to that necessary to drive the changes in the nervous system that we study. Surgical models of nerve damage most commonly involve very superficial nerves, with minimal surgery and nerve trunk damage. Chemotherapy models are all relatively

new compared with many other pain models. The models that we use are based on those with the most recently published refinements using the minimum chemotherapy dose necessary (often significantly lower than the equivalent clinical dose given to humans) to result in mild neuronal injury.

Why can’t you use animals that are less sentient?

Rodents are the least sentient species in which studies of this nature are performed. They are capable of decision making, which is completely necessary in assessment of pain. These attributes are not apparent in species such as amphibia and fish. Rats and mice have been extensively used in studies such as these, and so there is a great deal of published literature against which results can be compared.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Given that the focus of these experiments is mechanisms and treatment of pain, post-operative analgesia is not used. This is because all analgesics known either inhibit the initial activity generated by inflammation/damage that we wish to study, and that drives the development of chronic pain, or enhance nervous system mechanisms that also enhance or block pain mechanisms. This means that post-operative analgesia would interfere with the systems we are studying. We monitor animals frequently, particularly after surgical interventions, and ensure that their access to food and water is easy ie dishes placed on the cage floor, in case they experience some loss in mobility.

The models that we use have been extensively refined (often by us) over the years. As most of the work concentrates on the early changes that lead to chronic pain, the majority of studies do not last a long time, usually less than 1 month. Arthritis/inflammation/injury models are limited to a single joint/limb, and the mildest form of intervention suitable for the experimental aims is used. Models of nerve damage most commonly involve superficial nerves, with minimal necessary surgery.

Chemotherapy for cancer in humans also causes nerve damage, as it does in rodents and can also cause effects in other tissues. Chemotherapy models are all relatively new compared with other pain models; we keep abreast of published refinements, and implement these when appropriate for experimental aims.

We will, where possible and appropriate e.g. in study of pharmacokinetics of novel compounds on intra-articular injection, conduct the studies under terminal anaesthesia, thus reducing the impact on animal welfare.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

As above, the approach to this is the same as the approach to remaining informed about advances in the 3Rs. We consult colleagues on best practice, monitor information on best

practice produced by NC3Rs, UAR and other relevant Learned Societies and organisations, and discuss best practice with animal welfare colleagues who are informed of novel refinements and expectations through AWERB and Establishment Licence holder networks.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We consult colleagues on best practice and discuss refinements to models presented at seminars and conferences, and in published literature, including information produced by NC3Rs (e.g. best practice on mouse handling for minimal stress, and other resources in the NC3Rs resource hub), Understanding Animal Research and Learned Societies such as The Physiological Society and The British Pharmacological Society (e.g. validity and best practice use of the forced swim test). We discuss potential refinements with our animal welfare colleagues in the BioSciences Support Unit,

including the NACWO and NVS, who are often informed of novel refinements through AWERB and Establishment Licence holder networks. New refinements are discussed, and implemented when appropriate for the aims of the project.

182. The physiological roles of clathrin diversity

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:

Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Clathrin, Type 2 Diabetes, Immune cells, Cell and Tissue Development, Neurons

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Clathrin proteins are required for cells to function, and specialised forms of clathrin enable it to perform specialised activities in different tissues, including in cells that control metabolism, cells that regulate the immune response and nerve cells, amongst others. This project will define how the specialised components of clathrin (clathrin heavy chain (CHC) 22 and clathrin light chains (CLC) a & b) contribute to processes related to health and disease by establishing their function in animal tissues.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Clathrins contribute to a process called membrane traffic, whereby proteins move between the cell surface and the inside of the cell. Through this, clathrins regulate the ways by which cells communicate with each other and their environments. As such, clathrins play vital roles in many cellular functions, including, but not limited to, signalling between cells, tissue development and nutrient uptake. Given the importance of clathrins in so many cellular pathways, it is important to understand how they aid and alter different functions in different cells and tissue to allow clathrin to meet the membrane trafficking demands of a huge range of highly specialised cells.

Although the basic molecular mechanism of how clathrins function is well-studied, how they perform specialised functions in specialised tissues in the body is not yet established. Several different forms of each clathrin subtype (CHC and CLC) exist, and it is this diversity we propose as a mechanism to fine tune clathrin function in different cellular pathways in different tissues to achieve specialised functions.

In initial studies, we have already shown that CHC22 functions within the response to insulin in muscle and fat tissue, a key process to maintain steady blood sugar (glucose) levels. Furthermore, we have shown that common genetic variations within humans can alter this pathway, potentially affecting the rate at which glucose can be absorbed from the blood. Given the rise in patients suffering from Type 2 Diabetes, in which blood sugar levels become dysregulated, there is a fundamental need to understand the mechanisms of CHC22 function, and how these influence glucose balance in both a healthy and diabetic context. We hypothesise that, in the long term (beyond the scope of this project), a clear understanding of the mechanisms by which CHC22 influences blood glucose clearance may lead to the development of small molecules that specifically regulate CHC22 function in order to improve blood glucose regulation in diabetic patients.

In addition, we have already demonstrated that diversity of CLCs, in which humans and mice share the same six common variants, influences the functions of nerve and immune cells, as well as cells in the uterus. Our proposed work will continue these studies in animals to understand more about how CLC diversity affects tissue functions that play a role in metabolism, fertility, infection and brain function.

These studies will link molecular pathways with biological processes that determine health and disease, and thereby inform future preventative and therapeutic strategies. A potential example of this in the long term would be by helping to determine if clathrin pathways are possible druggable targets in cases of infertility.

What outputs do you think you will see at the end of this project?

The aim of this project is to understand how the different clathrin subtypes contribute to the diversity of clathrin function in different cell types and tissues. The project is split into two objectives, i) understanding the roles of CLCs during development of specialised cells and tissues and ii) uncovering the role of CHC22 in the development of Type 2 Diabetes. We

expect to have furthered our understanding of the functions of both CLCs and CHCs by the end of the project. The primary output of this research will be publications that are of interest to the fields of clathrin biology, membrane trafficking, neuronal development, organ development, glucose metabolism and diabetes.

Through using mice lacking specific CLC expression and mice expressing CHC22, we are able to provide tools to further study these proteins to the wider field. We will also disseminate our findings in seminars, conferences and the scientific journals.

Who or what will benefit from these outputs, and how?

Through comprehensively defining the roles of CLCs and CHC22 at the levels of the molecule, cell and organism, our work will benefit research in the field of clathrin biology and provide key missing links in our knowledge of how clathrin performs its critical cellular tasks. The findings and tools from this research programme can immediately be used by other researchers to inform on how clathrin diversity impacts in specific cellular pathways.

As CLC clathrins are involved in many essential biological processes, these studies will be of interest to a wide variety of researchers and, in the longer term, have the potential to provide important information leading to the development of treatments for a diverse range of diseases including, but not limited to, developmental defects, cognitive disease, infertility and cancer metastasis.

Our investigations into the role of CHC22 will help us to understand its function in a whole-body context and provide models to connect the properties of CHC22 identified in vitro to its function in glucose metabolism and diabetes in vivo. In the short term, therefore, those who will benefit most from this element of the research will be researchers in the fields of GLUT4 trafficking and glucose metabolism. Given that CHC22 has a limited expression pattern and is not involved the broader roles performed by other clathrins, it is an attractive target for therapeutic intervention. Therefore, in the long term, beneficiaries of this research include both commercial and academic groups interested in the development of treatments for diabetes, as well as people living with diabetes for whom novel therapies would be greatly valuable.

How will you look to maximise the outputs of this work?

We will actively seek to share our work in a number of ways. Manuscripts for publication that result from this work will be uploaded onto pre-publishing databases such as bioRxiv to ensure knowledge and findings are available to the scientific community as quickly as possible. In addition to publishing results in open access research journals, results will also be presented (prior to publishing) at relevant conferences and seminars, with the aim of both disseminating our findings and also receiving valuable input from fellow researchers and potentially establishing new collaborations. We already have ongoing collaborations relating to both projects, and we regularly meet to discuss results, findings and ideas that help inform our ongoing project directions. We have previously provided labs in several other countries with genetically altered (GA) mouse lines that we have generated in order

to maximise the potential scientific output from these animals. In addition, we routinely collect and store tissues and organs from our animals, which can be shared with the research community upon request.

In addition to presenting at scientific conferences, we are committed to maximising our outreach to the wider community. This will often be through outreach programmes such as Soapbox Science, as well as disseminating the findings of this research to the wider press after publication in scientific journals. In addition, we have established a connection with a Diabetic Patients Group and will discuss findings from the CHC22 mouse lines with this group in order to both guide future work and discuss the best ways to disseminate our work more widely.

Species and numbers of animals expected to be used

- Mice: We estimate to use approximately 5000 mice over 5 years.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

In order to study the role of CLCs in tissue development, we require an animal model that both closely resembles human biology and expresses homologues of all the human isoforms of the CLCs.

Therefore, we require a mammalian animal model, since mammals express all CLC isoforms with greater than 90% identity. We have chosen mice as our model animal because their biological and genetic characteristics are very similar to those of humans and because the range of tools available to alter their genetic code makes them suitable animals for our project.

CHC22 is a pseudogene in mice, which means that mice do not express CHC22. We are using mice, therefore, as it allows us to investigate how CHC22 regulates blood glucose balance in a biological context which is very similar to humans' but where CHC22 is usually absent. We have observed that the expression of CHC22 in mice leads to the development of a mild diabetic trait. We therefore will use these mice to investigate the link between CHC22 and Type 2 Diabetes.

As we want to understand the role of different CLC isoforms during the tissue development process, we need to examine animals at all ages in order to identify the age of onset of abnormalities. This requires careful examination of embryonic mice, pups and adult mice. In addition, we have observed characteristics in CLCa KO mice that only appear in animals over 4 months of age, suggesting age influences the tolerance of some cells and

tissue to the absence of CLCa. Thus, we will examine older mice in order to identify which biological processes are affected as GA mice age.

Additionally, we want to know how CHC22 function influences blood sugar levels and how the failure of this process is linked to Type 2 Diabetes. As Type 2 Diabetes is associated with age, we will use adult mice for this objective.

Typically, what will be done to an animal used in your project?

GA mice will be bred by trained and competent animal technicians employed by the local animal housing facility, where they will be closely monitored by animal house staff and personal licence holders. For CLC GA animals, most mice used will be humanely killed at various life stages without being subjected to any other procedures. Tissues from these animals will be examined by microscopy for differences between unaltered (wild-type) animals and their GA littermates, in order to identify how the loss of CLC impacts protein expression and localisation, cellular functions and tissue development. Mice may be subjected to minor procedures that do not cause lasting harm, such as injection of non-toxic substances in order to examine cellular function, before being humanely killed and examined.

CHC22 mice will be fed differing diets before being subjected to glucose/insulin tolerance tests, in which blood is drawn at regular time intervals following stimulation by intravenous injection of glucose and/or insulin, in order to examine the link between CHC22 function, diet and diabetes. These minor procedures are well established and do not cause distress to the mice.

What are the expected impacts and/or adverse effects for the animals during your project?

Our protocols do not result in severe pain, distress, or lasting harm. We will breed GA mice that display mild/moderate lasting characteristics. We expect a 50% post-natal (1-3 days after birth) death rate of CLCa KO animals, indicating a critical role for CLCa in development. We do not observe mice fading away, suggesting these mice die very quickly, without prolonged suffering. The surviving mice are slightly smaller than non-GA animals, with on average 5-10% weight loss. In addition, older CLCa GA female mice have a higher risk of developing uterine infections, as shown by an enlarged abdomen.

The CHC22 mice tend to have slightly higher blood sugar levels. Most of our experiments will be performed in order to identify the changes caused by genetic alterations. In some experiments, animals may experience mild anxiety resulting from high/low blood sugar. Some of our protocols require that we collect organs for analysis, so mice will be given terminal anaesthesia before being euthanised via approved methods. At the end of our studies, mice not used in further experiments will be humanely euthanised.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Just under half of CLCa KO mice develop enlarged uteri, which means that this line is categorised within the moderate category. This characteristic is only seen in CLCa KO animals (and not mice that lack only one of the two copies of this gene). Given that approximately half of these KO animals die 1-3 days after birth, this means we expect <15% of surviving CLC mice to be this category. CLCb KO mice have no defining negative traits, and thus are classified as mild (75% of CLCb contain a loss of either one or both copies of this gene). We expect 50% of CHC22 mice to have one copy of the gene introduced to their genome. The only detected characteristics of these animals are a mildly elevated blood glucose level, and therefore these animals are categorised as mild.

What will happen to animals at the end of this project?

- Killed
- Kept alive
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

While we address a lot of our research interests using in vitro methods, a key aim of our work is to understand the impact of clathrin diversity on tissue development as well as disease. We are unable to do this using in vitro techniques alone, as it is not possible to fully recapitulate complex tissue structure in cell culture. As interactions between the different cell types that make up a tissue are critical to tissue development, we require animal models to fully understand the roles of clathrin diversity. Specifically, we need to add (CHC22) or remove (CLCs) the different types of clathrin in order to directly compare the different cells and tissue in these mice to their wild-type equivalents. This will direct our focus to tissues that are specifically affected by altering clathrin expression, and at which developmental stages these abnormalities occur. Overall, these animals will provide us with vital information on the critical roles of the CLCs and CHC22 that it is impossible to obtain without the use of mice.

Which non-animal alternatives did you consider for use in this project?

We will mostly use cultured cell lines, not animals, for our work. Although we will use mouse models in some of the experiments described in our project, we always first attempt to answer our research questions using cultured cells or biochemical experiments. For example, we have begun using the latest techniques in murine organoid cell cultures

(cultures of cells grown in the lab that more closely resemble the structure of an entire organ than single cell culture alone). We have established intestinal and endometrial (uterine) organoids from cells isolated from our GA mice. Now these organoids have been established, they can be grown indefinitely and cryopreserved, greatly reducing the numbers of mice we need to use in our research through replacing the use of further animals with these in vitro systems to study this tissue.

Why were they not suitable?

Traditional in vitro cell culture has been critical to establishing clathrin's multiple functions within the cell. However, since these methods typically involve single immortalised (usually cancerous) cell lines, they are not able to inform us on how clathrin's functions influence the cross-talk between normal (non- cancerous) cells types in vivo that is vital to tissue development. While organoids represent a positive step forward in this regard, and enable us to study multiple cell types in vitro, they still do not fully mimic organs and tissues in vivo because they do not encounter hormones and nutrients in the same way as an organ in the body. Thus findings using organoids should ultimately be verified using appropriate animal models and studies in animals will reveal behaviour of tissues in context. In addition, organoid culture techniques are still relatively new, and there are many organs for which we are not able to produce representative in vitro organoids.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimation is based on our previous experiences and our future experiment plans. Many animals will be used for breeding but not in other experiments. Due to <15% of adult mice are CLCa KO, rather than the expected 25%, due to post-natal lethality in the first 7 days after birth, a higher number of CLCa GA mice are required for breeding to produce sufficient CLCa KO mice for our requirements.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

POWER calculations were used to establish the maximum statistical power for the minimum number of animals possible. In addition, we will perform pilot studies in order to establish single experimental doses and time points for our experiment. This greatly reduces the number of animals used by minimising the number of experimental groups.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Since all protocols and procedures used in this study cause no more than moderate (usually only mild) and transient discomfort, mice will be used in multiple protocols where possible in order to reduce the total number of mice used.

In addition, we will reduce the number of animals used by performing pilot studies in order to establish single experimental doses and time points examined, and therefore minimising the number of experimental groups required.

We continue to collect and freeze the tissue from euthanised mice, for long term storage of the tissue we do not examine at the time. This maximises the potential use of the tissue of each mouse, thereby reducing the total number of mice required.

Further to this, we continue to aim to incorporate new in vitro techniques as they are established into our research in order to reduce the number of mice required to meet our experimental aims.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are interested in how biochemical diversity of clathrin generates differential function at the cellular, tissue, and organism levels, and how this relates to human disease (e.g. diabetes). Mice are ideal for our studies as their genetic background and tissue organisation is similar to humans. The majority of our mice will be used for histological and biochemical analysis of cells and tissues, where mice are anaesthetised and killed and, as such, do not experience further pain or distress. Some mice may be used for procedures that cause no more than mild/moderate and transient distress, such as a single dose injection or glucose/insulin tolerance tests. These are the most appropriate systems for our questions, and we will only use mice when the experimental question at hand specifically requires them.

Why can't you use animals that are less sentient?

We have explored using Zebrafish (Danio rerio) and flies (Drosophila melanogaster) instead of mice, however flies only express a single CLC isoform, and so are not a

sufficient representation of human genetics to be useful for our investigation, and zebrafish do not sufficiently mimic human tissue development for our requirements. Mouse and human biology are more similar, and thus will be more informative as to the functions of clathrin in human tissue development and disease.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will always aim to minimise pain, suffering, distress, and lasting harm in the animals we use. We only propose experiments entailing mild/moderate distress. We will use analgesia and anaesthesia where possible, and have pre-determined humane end-points in place for all of our experiments. We will monitor our mice consistently and carefully, noting unexpected traits of GA mice, and stop procedures immediately if those end-points are reached. In particular, we will monitor female CLCa KO mice over the age of 4 months carefully for the appearance of a swollen abdomen to minimise the harm caused to these animals. In addition, we will perform health assessments regularly and before each experiment. This is important both to reduce suffering in our mice and to ensure scientific best practice for the reliability of our results. When handling mice, we will practice non-aversive mouse handling techniques to reduce the stress of mice, and we will only use each needle once when performing injections.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will practice the general guidelines and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines published by NC3Rs (https://www.nc3rs.org.uk/3rs-resources and https://arriveguidelines.org). We will also use systematic reviews, such as SyRF (Systematic Review Facility), to ensure we are using the most appropriate methods to minimise suffering, as well as using the fewest possible number of mice.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will utilise the NC3R’s (National Centre for the Replacement, Refinement and Reduction of Animal in Research) website for up-to-date information in the best practices in refining and reducing animal models. We will continue to attend training courses for animal use and we will be informed and trained in up-to-date techniques by the NTCO, NVS and NACWO.

183. Breeding and maintenance of genetically altered mice

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Breeding, Maintenance, Mice, Genetically altered

Animal types	Life stages
Mice	embryo, neonate, juvenile, pregnant, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To produce, maintain, and provide genetically altered mice for use under other project licences, or direct supply of tissue/organs for ex vivo use.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Notwithstanding the fact that human and mouse genomes have been fully sequenced and studied in detail in recent decades, the function of many genes, and their involvement in health and disease, is still unknown. Using a range of sophisticated technologies, the DNA

of mice can be safely manipulated to allow for experiments that elucidate the role that candidate genes play in a range of biological processes. In turn, these insights can lead to treatments that can prevent or treat disease and optimise the health of both animals and humans.

This licence application (and previous versions of the licence) is intended to provide an efficient service for breeding genetically altered mice and transferring them to researchers for further study, either as whole animals for use on other project licences, or tissues for use in ex vivo studies. The importance of each mouse colony and the benefits of each project will be described during a formal request process, submitted and reviewed by the Animal Welfare & Ethical Review Board (AWERB), described elsewhere in this application. In recent years, successful applications have been received to launch or continue projects in the following research areas: lung function & disease, chronic pain, cancer, neurodegenerative diseases & cognition, and muscular disorders.

What outputs do you think you will see at the end of this project?

The primary output from this licence will be the supply of genetically altered animals to research groups within, or affiliated to, the University. Use of these animals on other in vivo and ex vivo projects will result in data that will be shared within the scientific community at conferences, symposia, and in publications (these will be summarised at the end of the project).

Who or what will benefit from these outputs, and how?

As mentioned in the 'Aims' section ('Why is it important to undertake this work?'), despite human and mouse genomes being fully sequenced, the function of many genes and their involvement in health and disease is still unknown. This project will benefit people and animals by revealing the genetic contribution to a range of biological processes that are involved in health and disease. Where genes play a role in disease processes, this may provide better diagnosis and/or earlier and better treatment of a disease. Specific benefits will be described in those projects to which animals and tissues will be supplied.

A list of academic outputs from the last five years are provided elsewhere in the application. A brief outline of research areas and how animals have contributed is provided below.

Lung function & disease: the involvement of particular genes in biological mechanisms that underly chronic obstructive pulmonary disease (COPD); the protective effect some genes have over developing pulmonary diseases associated with old age, such as fibrosis.

Chronic pain: vascular and inflammatory contributions to pain disorders, especially those caused by diabetes and arthritis.

Cancer: discovery of novel mechanisms by which cancer cells display resistance or tendency towards metastases; further identification of upregulation of genes in different

types of cancer; how the effectiveness of chemotherapy may be improved in some types of cancer by altering gene expression in tissues that surround the tumour.

Neurodegeneration: characterising behavioural abnormalities in animal models of neurodegeneration; investigating inflammatory mechanisms underlying neurodegenerative diseases and genes that may have protective effects; identifying imaging markers that track and predict disease progression.

Muscular disorders: identification of a therapeutic target to treat muscular disorders.

The benefits of concentrating colony management under a single, central licence are described fully elsewhere but, briefly, this approach allows our technical team to carefully manage every colony to produce high quality animals in numbers that closely match demand. This means the research carried out subsequently will be more likely to show reproducible, useful results and animal numbers are kept low. Centralised management also more easily permits excess animals to be identified and shared to the scientific community, ensuring as many animals as possible contribute to the advancement of knowledge.

How will you look to maximise the outputs of this work?

In accordance with the Home Office's 'Efficient Breeding of Genetically Altered Animals Assessment Framework' - and where confidentiality agreements allow - we will advertise colony details to all research groups to allow for collaboration and cost-sharing. This also helps to minimise wastage.

Species and numbers of animals expected to be used

- Mice: 37500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are biologically very similar to humans and can be genetically manipulated to mimic many pathological conditions, which is not the case for non-protected alternatives such as fruit flies. As this is a breeding licence, all life stages < 15 months may be used (i.e. all stages except for aged animals).

Typically, what will be done to an animal used in your project?

Mice only enter our breeding facility upon satisfactory completion of our internal AWERB review process and verification that mice are of a high health status (described in the Action Plan, 'How do you assure the quality of the products?'). Mice will be initially bred

according to the strategy finalised during discussions between technical staff and the research group in advance of animals arriving. Most animals will not experience any other procedures whilst on this licence; animals will be transferred to other licences or humanely killed, some via a non-schedule 1 technique such as perfusion. Most animals bred will be identified by taking an ear notch and the tissue used for genotyping.

What are the expected impacts and/or adverse effects for the animals during your project?

No mice are expected to have a harmful phenotype. If this is anticipated for new colonies, an amendment to the licence will be submitted.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

The majority of animals to be bred under this licence will expect to be classified as 'sub-threshold'. Animals reported as having experienced 'mild' severity are likely to have undergone a second tissue sample (solely for genotyping) or display a distinct but non-harmful phenotype. Based on previous experience and knowledge of the strains used in research at this establishment, more than half of all animals to be bred under this licence would be expected to have a severity classification of 'sub- threshold'.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This is a service licence for the breeding and maintenance of genetically altered mice. The scientific aim(s) of individual breeding programmes are described in formal breeding requests that are submitted to AWERB, and only after satisfactory review (described elsewhere) would a programme commence. Observations based on live animals are necessary because understanding the overall effects of a modified gene requires the interaction of multiple physiologically-intact organ systems.

Which non-animal alternatives did you consider for use in this project?

Researchers requesting that mice be bred under this licence must describe the non-animals steps that have preceded the in vivo stage. AWERB will determine that animal use is justified by confirming that, as far as is practical, all non-animal steps have been completed. For instance, and where relevant, studying the impact of genetic alterations in vitro (in cells) before progressing to genetic alteration in animals. Furthermore, researchers must explicitly explain to AWERB why non-animal methods are unsuitable.

Why were they not suitable?

Reasons why non-animal alternatives are not suitable will be assessed on a case-by-case basis during AWERB review; broad justification of the use of animals under this licence is provided above. Where animals are being supplied for use in other projects, reasons why non-animal alternatives are not suitable must correspond with reasons provided in the relevant project licence and will be confirmed during AWERB review.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The estimate is based on the annual numbers of animals bred under previous versions of this licence. A turnover of colonies comparable to previous years is anticipated but numbers required to achieve the scientific aims of each individual colony varies. Robust colony management practices are employed to maintain efficient breeding programmes (described elsewhere) and these are reviewed and clarified with researchers as each new line is introduced.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

During a research group's application to breed and maintain mice under this licence, the requester is expected to describe the experimental design and this is interrogated during the AWERB review (see below). AWERB and the PPL holder expect to see good practice guidelines being followed such as: minimising background variability in the data; high variability can obscure results which may require additional animals be used to reveal differences between experimental groups (there are several ways to do this such as eliminating unconscious bias by blinding researchers to which experimental group an animal belongs) a description of how the required number of animals has been determined, including justification of key design parameters such as 'effect size' (a parameter that indicates how large a difference between experimental groups a researcher considers meaningful) where possible and appropriate, incorporating the sex of mice into

the experimental design; this ensures more mice from a litter can be used, provides additional data regarding potential sex differences, and can often be performed - with careful choice of experimental design - without an overall increase in animal numbers

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Animals are maintained at a high health status by the use of appropriate biological containment, typically the use of individually ventilated cages (IVCs) within a centralised barrier facility. This will reduce the microbiological burden on the animals and lead to a reduced disease incidence and reduced variability in experimental results.

In accordance with the Home Office's 'Efficient Breeding of Genetically Altered Animals Assessment Framework', we will hold regular reviews of colonies in production. This is to ensure that breeding strategies are optimised to achieve the delicate balance of generating the correct genotype and number of animals each research group require, minimising unnecessary breeding, and maintaining sufficient stock to not endanger the survival of a colony. Where research objectives have been met and no short/medium term demand is anticipated, colonies will be either archived or production stopped (typically within 12 months).

The above Home Office Framework dovetails with best practice recommended by the NC3Rs' expert working group on breeding and colony management, which we will also observe where appropriate. These provide practical guidance on elements of the framework that can help reduce numbers of animals used, such as:recognising reproductive life cycles to optimise breeding efficiency and health of litters
recording breeding data to ensure relevant information is captured that can help identify changes in breeding performance and expedite important colony management decisions
refreshing a colony to avoid 'genetic drift', a process that results in colony 'drifting' away from the original genetic identity that can reduce the reproducibility of studies

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will produce, maintain, and provide mice for use in other projects that have successfully completed the AWERB review process (described elsewhere). These mice

constitute animal models of a range of different diseases or disease processes; in recent years, these have included models of lung disease, chronic pain, cancer, neurodegenerative diseases, and muscular disorders. Animals are not expected to exhibit any harmful phenotype and procedural steps are not expected to cause any adverse effect for the animals that is more than mild and transient.

Why can’t you use animals that are less sentient?

As mentioned in the 'Harms' section, mice are biologically very similar to humans and can be genetically manipulated to mimic many pathological conditions, which is not the case for non-protected alternatives such as fruit flies. Nevertheless, as this is a service licence, the breeding request form will ask that requesters explain why their particular area of research cannot utilise non-protected alternatives or less sentient protected animals such as zebrafish. Mice will only be bred if appropriate justification is provided and the request has successfully completed the AWERB review process.

Depending on the genetic alteration and area of research, not all mice may be required to develop into adulthood but it is anticipated that the vast majority will. The only part of the project for which terminal anaesthesia may be appropriate is for non-schedule 1 forms of humane killing, for which authority is sought in the relevant section.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Mouse passports are required during the AWERB review process so that the requirements of every new colony can be fully understood and, if necessary, husbandry practices adapted so that we can optimise their care and minimise harms. Upon receipt of a new colony or additional mice for breeding, a thorough welfare assessment is always performed.

Animals are not expected to exhibit any harmful phenotype and procedural steps are not expected to cause any adverse effect for the animals that is more than mild and transient. All technical staff undergo thorough training to ensure they are proficient in daily husbandry duties and adept at identifying any potential welfare concerns. Carefully constructed Standard Operating Procedures (SOPs) and, for practical assessment, Directly-Observed Practical Skills (DOPS) sheets are used within our Unit for these purposes.

We also adhere to best practice guidelines (listed below) and group house colonies as standard, utilise non-aversive handling (which the PPL holder has promoted at national events), and ensure environmental enrichment is present in all cages. Attendance at training events and networks (mentioned above) ensures that staff are made aware of any suitable refinements for the breeding and maintenance of genetically altered mice.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The technical team and PPL holder periodically review working practices against the following guidelines:

'Efficient Breeding of Genetically Altered Animals Assessment Framework' (Home Office)

Materials produced by the NC3Rs' 'Expert Working Group for mouse breeding and colony management' and disseminated via the Breeding and colony management hub (NC3Rs)

Welfare assessment recommendations from 'Assessing the welfare of genetically altered mice' (Wells et al. 2006, Lab Animals)

Our University also publicly supports and endorses the ARRIVE guidelines and researchers requesting use of this licence must confirm they will be used and are directed towards appropriate online guidance.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The applicant is the co-organiser of the Regional NC3Rs Symposium and attends other similar regional events to remain up-to-date with advances in the 3Rs. The 'Experience' section lists formal training events the applicant has previously attended and delivered. The technical team also regularly attend training events (supported or delivered by, for example, the NC3Rs, IAT, RSPCA, and LASA) to ensure knowledge is up-to-date and broaden their networks to allow the sharing of best practice. The University also benefits from an NC3Rs Regional Programme Manager to further embed the establishment within wider 3Rs networks.

184. Glymphatic system in the ageing brain

Project duration

3 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

brain, cerebrospinal fluid, glymphatics, ageing

Animal types	Life stages
Rats	adult, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to characterise the inflammatory and fibrotic changes in the glymphatics system that occur with ageing.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The cerebrospinal fluid (CSF) dynamics system is important for maintaining brain homeostasis. Part of this is the glymphatics system, which ensures efficient fluid clearance from the brain. Impairments in this system have been observed with ageing and have been

associated with age-related diseases such as Alzheimer's disease. Changes have also been observed after acquired brain damage following traumatic brain injury and stroke (ischaemic and haemorrhagic). However we do not fully understand what changes in the brain contribute to alterations in the glymphatics system.

Advancing our knowledge of age-related changes that occur in the glymphatics system could identify a therapeutic target for treating age-related neurological disorders or limiting the damage of acquired injury in older patients.

What outputs do you think you will see at the end of this project?

The main output from this project will be improved fundamental understanding of the age-related changes that contribute to impaired glymphatics and the importance of inflammatory and fibrotic mechanisms.

These outputs will be published in high impact peer-review publications and the data will be used for future grant applications to progress glymphatic research.

Who or what will benefit from these outputs, and how?

In the short term (<5 years) the main beneficiaries of this research would be the academic community. This project will impact researchers interested in inflammation, CSF dynamics, glymphatics, ageing and neurological disorders (hydrocephalus, dementia and stroke).

In the medium term (5-10 years), our outputs will provide fundamental discovery science regarding how the glymphatics system is affected by inflammation and fibrosis that occurs during natural ageing. This will provide the necessary background information to bridge between basic science and identification of potential therapies.

In the long term (>10 years) this research has the potential to identify inflammatory and fibrotic mechanisms that can be targeted therapeutically to improve the outcome for patients with cognitive decline due to ageing, neurodegenerative diseases and acquired brain damage. Therefore, patients and clinicians could benefit from this fundamental research. As many of these disorders are associated with significant disability it requires substantial support from the NHS (and other healthcare providers)

and their families or carers. Therefore, new treatments could improve the outcome of these patients have a positive financial impact on the economy by reducing healthcare costs and allowing family members who are carers to return to work.

How will you look to maximise the outputs of this work?

The results from this study will be important for advancing our knowledge around the ageing, CSF dynamics and the glymphatics system. The results will be disseminated in peer review publications and by social media, whether the results are positive or negative. Publication of negative results are just as important to prevent other researchers from investigating the same thing.

Species and numbers of animals expected to be used

- Rats: 100

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Previous glymphatics research has involved rats and mice, and both have been shown to be suitable for assessing glymphatics function using tracers. Rats are larger and have easier access to the glymphatic system for tracer injection and hence fewer adverse events. In addition, there are subtle differences in CSF flow dynamics due to the effect of the larger perivascular spaces in rats compared to mice, which offer less hydraulic resistance to CSF flow. Performing our experiments in rats is therefore more translational and our familiarity in rat models is essential to making timely progress. Aged rats start to show cognitive dysfunction and alterations in the CSF dynamics system after 18 months which is why we have chosen the age range of 18-20 months for our aged animals.

We will also be using young (2-3 month old) rats as a comparison for the age-related changes to CSF dynamics and the glymphatic system.

Typically, what will be done to an animal used in your project?

Some animals will be aged to 18-20 months. All animals will undergo a procedure which is performed under non-recovery anaesthesia. The animals will receive a tracer injection into the cisterna magna, be kept under terminal anaesthesia for up to 1 hour and humanely killed at different time-points during this time, via a schedule 1 method.

What are the expected impacts and/or adverse effects for the animals during your project?

There may be some adverse effects in the ageing cohort such as behavioural changes, weight loss, or the development of tumours.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Around half of the animals will undergo a procedure conducted under terminal anaesthesia and therefore will experience non-recovery severity. The rest of the animals will be aged and so will experience mild severity.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Whilst some elements of CSF dynamics and the glymphatic system can be modelled in cell culture, the complex, clinical picture and interaction of the whole-body systems, including in particular, the immune and nervous systems cannot all be currently modelled in cell-culture or computer-based models. The use of live animals is therefore unavoidable and essential for discovery science in a situation relevant to the human condition. Only mammals have a sufficiently developed immune-system to readily compare to humans, and rodents are the animals of lowest neurophysiological-sensitivity required to achieve our scientific aims. It is not ethical to conduct experiments on humans, especially where those experiments require the removal of parts of the immune or nervous system for ex-vivo investigations.

Zebrafish or other lower sentient animals cannot be used since these species spontaneously regenerate their nervous system and are therefore not representative of the human conditions of disease that aberrant CSF dynamics and glymphatic system cause. Therefore, there is no feasible alternative that can entirely replace the use of a living animal that would allow the objectives to be met. However, we will use in vitro and ex vivo work to inform our animal studies.

Which non-animal alternatives did you consider for use in this project?

There are currently no alternatives to animal work for CSF dynamics and the glymphatic system.

No cell culture-based models exist that encompass all of the aspects of CSF/glymphatics. However, individual aspects will be modelled in vitro and ex vivo. For example, we regularly use in vitro approaches using partial replacement of animals (primary animal cells) or non-animal alternatives (primary human cells) can be used to assess the effects of inflammation and fibrosis on the cells of the glymphatics system.

Why were they not suitable?

The fundamental reason why the use of animals is required is to understand these complex processes that occur in whole animal, which at present no in vitro methods can model the complexities of the systems involved in these systems. It is difficult to use primary cells to culture all of the different types of cells since they require different growth mediums and factors for survival. Indeed, the reason why many new drugs fail between cell culture and in vivo studies is in the inability to full recapitulate the in vivo environment.

Technologies are being developed to address this gap, including the development of 3D cultures and organoids. However, none of these model systems are yet able to replicate the integration and interplay between the numerous cell types that constitute CSF and glymphatic systems.

Reduction

How have you estimated the numbers of animals you will use?

The experiments have quantitative endpoints and an estimated number of 8 animals per group has been established as the minimum required to provide statistically significant results. This has been determined on the basis of previous research into ageing and the glymphatics system.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We use the NC3R's Experimental Design Assistant to help us to design the experiments to ensure that the experiment uses the minimum number of animals but that it is sufficiently powered to achieve our scientific aims.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will seek to refine protocols, such as the development of other quantitative measures for CSF dynamics/glymphatics such as associated biomarkers that can predict potential age-related changes, which will facilitate “reduction”. Experiments will be planned so that they can be published in accordance with the ARRIVE 2.0 guidelines.

At the end of the experiment, we will harvest the maximal possible number of tissues. Tissues not immediately analysed will be archived and will be made available to other researchers working on similar questions.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Due to the nature of the scientific question, the only appropriate model is young and aged rats. The method of assessing glymphatic function involves injecting a tracer into the cisterna magna and is the most commonly used method. The procedure is conducted under non-recovery anaesthesia to minimise the pain and suffering of the animals.

Even though, all experiments will be performed under terminal anaesthesia, we also have over 20 years' experience of similar models and injections described in this application and are able to identify adverse signs as early as possible.

For aged rats, we have selected 20 month old rats for our experiments since this is the earliest timepoint where changes in the glymphatic system are measurable rather than a greater lifespan where increased age-associated adverse effects are more evident.

Why can't you use animals that are less sentient?

We cannot use less sentient species (e.g. zebrafish) for this work, because unlike mammals, they are able to regenerate their brain tissues spontaneously and so changes observed as a result of dysregulated CSF and lymphatics are not seen. Rats are typically used for our experiments since they share similar pathophysiology to humans after injury to the CNS and share similar dynamics of CSF flow and glymphatic systems.

In addition, we are using animals that have been terminally anaesthetised and hence potentially minimising adverse effects.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Animals in the aged cohort will be closely monitored, which increases in frequency when the animals get older (>18 months). While the animals are ageing they will have access to additional enrichment toys and access to a play pen.
This protocol involves the minimum number of interventions possible for assessing glymphatic function. In addition, all animals are terminally anaesthetised and hence unnecessary harm is minimised.
We will review each experiment on completion to see what lessons can be learned from the study in terms of endpoints.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Prior to all experiments we will consult the PREPARE guidelines checklist to ensure that valuable data will be generated in the experiment.

Experiments will be conducted in accordance with the guidelines published by the Laboratory Animal Science Association (LASA).

The resulting data will be published in Open Access Journals wherever possible and in accordance with the ARRIVE 2.0 guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will review each experiment on completion to determine any refinements that can be applied to future experiments.

Continued review of the scientific literature will be undertaken on a regular basis in order to identify any newly emerging technologies and models that could be potentially adopted in order to replace in vivo animal use.

We will also stay up to date with guidance published by LASA on the most refined experimental methods. We are already signed up to receive the NC3Rs newsletter and will attend local events such as conferences and follow advice in webinars hosted by NC3Rs.

185. Immune responses in tumour development

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cancer, immune cells, immunotherapy, tumour, myeloid cells

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We aim to examine how modulation of immune cell activity can enhance tumour suppression. The proposed research will focus on the assessment of interactions between tumour cells and immune cells that orchestrate anti-tumour immunity to better understand the immune mechanisms that shape tumour growth.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Certain types of immune cells are able to recognise, kill and remove ‘foreign’ material that grows within a host. However, tumour cells have developed ways to evade the immune system, exploit immune cell functions and compromise anti-tumour responses. Therefore, the development of strategies that aim to educate the immune system for enhanced tumour killing is needed.

Tumour immunotherapy represents a promising therapeutic approach for several types of cancer. However, some cancer patients receiving immunotherapy show limited or no response. This work will investigate how reprogramming of immune cells can increase the efficacy of tumour immunotherapy, thus addressing a timely and unresolved scientific question.

What outputs do you think you will see at the end of this project?

Successful completion of this project will result in the generation of data that will provide a better understanding of immune cell-mediated mechanisms of tumour suppression. These findings will be presented to the scientific community via peer-reviewed publications and at national and international meetings.

Who or what will benefit from these outputs, and how?

Short-term (0-5 year): Identification of immune modulatory agents and mechanisms that prime immune cells to enhance anti-tumour activity in mice and in humanised mice (mice that allow human immune cell production).

Medium/Long-term (5+ years): Findings of this research may benefit cancer patients and clinicians using immunotherapies, especially in oncology

How will you look to maximise the outputs of this work?

Collaboration with experts in regulation of immune cell activity and tumour development in both mouse and human settings will enhance the outputs of this project.

Additionally, any negative data produced in this project will be published in order to share knowledge and avoid repetition of the experiments by other research groups.

Species and numbers of animals expected to be used

- Mice: 1700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Laboratory mice have been extensively studied in the field of tumour immunology and represent the best animal model for this type of study. As this project focuses on the role of immune cells on the modulation of tumour suppression, adult mice, that have a developed immune system, are required. Humanised mice will also be used in order to improve the translational impact of the research.

Typically, what will be done to an animal used in your project?

Administration of substances (up to 2 times) that will modulate immune cell function followed by isolation of cells to examine them in vitro. Cell isolation will take place 1-30 days after administration of substances.

Some animals will be given identified substances plus/minus current immunotherapy drugs followed by tumour cells, and tumour growth (up to 10-12 weeks) will be monitored (via imaging) to examine the effect of the substances on tumour rejection.

Finally, some animals will be irradiated and will be transplanted with cells before their injection with substances and / or tumour cells.

What are the expected impacts and/or adverse effects for the animals during your project?

Mice will develop tumours.

In some cases, mice will demonstrate mild or moderate but temporary discomfort. Examples include mild and short-term discomfort due to injections or restrain, and short-term mild or moderate symptoms related to tumour cell injection (e.g. hunched posture, and slightly reduced level of activity). In addition, tumour growth may lead to weight loss of the animals.

Hypothermia may occur during in vivo imaging. Therefore, temperature monitoring and controlled heating during imaging will be used.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

30% of animals will only receive injection with substances, and will experience mild severity.

70% of animals will receive injection with substances and tumour cells, and may experience moderate severity.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project focuses on the role of immune modulators in shaping anti-tumour activity. Production of immune cells in the bone marrow is a major determinant in the induction of anti-tumour activity.

Therefore, the use of animals is necessary for the characterisation of immune cells in bone marrow, periphery (spleen) and at the tumour site as a result of immune modulator administration.

Which non-animal alternatives did you consider for use in this project?

Search at the Animal Welfare Information Centre (listed in NC3Rs website) has been used to provide alternatives.

In addition, in vitro research will include the stimulation of immune cells with immune modulators in the presence or absence of tumour cells using cell culture. This alternative is expected to be used to inform

in vivo work.

Why were they not suitable?

The use of in vitro cell culture does not allow simultaneous assessment of the interactions between different tissues (bone marrow, spleen, tumour) that take place in a whole animal model and therefore has limited physiological relevance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Animal numbers were estimated based on previous experience with the types of experiments proposed in this project and published datasets.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Quantitative analysis of findings obtained from previous research projects and information available through collaborators and from the literature were used.

Freezing of tissues of interest creates tissue bank to test hypotheses without need for new animals. In addition, mathematical and computational tools have been applied.

Experimental design will be supported with NC3Rs Experimental Design Assistant

(https://www.nc3rs.org.uk/experimental-design-assistant-eda) and results will be published according to

ARRIVE guidelines.

Statistical advice sought from Hull York Medical School statistical consultancy service (http://www.hyms.ac.uk/research/resources-for-researchers/statistical-consultancy).

Planning of the in vivo research will be based on the PREPARE guidelines (https://norecopa.no/prepare) will be used to plan in vivo research.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

When required, pilot experiments will be performed to generate data to calculate the number of mice required to obtain meaningful, reliable results. Small pilot studies will also be used to validate the effect of novel compounds or techniques before performing larger experiments.

Best practice for pilot studies will be used ((https://www.nc3rs.org.uk/conducting-pilot-study)).

In addition, the analysis of as many organs as possible from each mouse and sharing tissue with in- house collaborators will reduce the number of mice required for pilot studies, and maximise the output of data per mouse.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use analysis of tumour growth by in vivo imaging. The model used will be adult mice. In all cases, we will use the most refined ways to induce modulation of the immune system and tumour challenge models required to arrive at meaningful results. These ways of immune modulation are in general very well tolerated by mice with only mild to moderate transient discomfort. In all cases, strict monitoring and humane end points will ensure maximum reduction of pain, suffering, distress, or lasting harm to the animals.

Why can't you use animals that are less sentient?

As this project focuses on the role of immune cells on the shaping of anti-tumour activity, it is critical that adult subjects are used in the experiments, so that immune cell production and differentiation is developed in both bone marrow and in the periphery (spleen).

Laboratory mice have been widely used for the study of tumour immunology. Of note, research studies applying injection of mouse tumour cell lines in mice have contributed significantly to the development of immunotherapeutic approaches. In addition, high availability of genetically-engineered animals and reagents further supports the utilisation of mice in comparison to other laboratory rodents.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The welfare of animals will be frequently monitored, and a detailed welfare assessment scoring system will be applied. The grimace scale will also be used to monitor quality of animals' life. Whenever applicable, use of anaesthesia will minimize distress. In addition, enriched housing, tube handling and single-use needles will be used.

In vivo imaging may be used to identify end points allowing the S1K of animals before they show clinical signs of tumour progression.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

PREPARE guidelines for experimental planning:

Smith, AJ, Clutton, RE, Lilley, E, Hansen KEAa, Brattelid, T. (2018): PREPARE: Guidelines for planning animal research and testing. Laboratory Animals, 52(2): 135-141.

https://norecopa.no/PREPARE

NC3Rs refinement info: https://nc3rs.org.uk/3rs-advice-project-licence-applicants-refinement

The guidelines for the welfare and use of animals in cancer research published by Workman et al will

be considered (https://www.nature.com/articles/6605642).

ARRIVE guidelines will ensure experiments are performed in the most refined way. Additionally, we will follow the updates by the NC3Rs (https://www.nc3rs.org.uk).

The Laboratory Animal and Science Association (LASA) regarding the routes of administration of immunomodulators and transplantation of cells (https://www.lasa.co.uk/PDF/LASA- NC3RsDoseLevelSelection.pdf), the BVAAW / FRAME / RSPCA / UFAW Joint Working Group on

refinement (Laboratory Animals 2001. 35:1-41) and the IQ 3Rs Leadership Group (https://iqconsortium.org/images/LG-3Rs/IQ-

CRO_Recommended_Dose_Volumes_for_Common_Laboratory_Animals_June_2016_%282%29.pdf)

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The host institute and establishment organise mandatory and frequent training as well as expert users' forums with the latest advances in the 3Rs.

Additionally, we will follow the updates by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and by the Laboratory Animal and Science Association (LASA) newsletters.

186. Central nervous system control of physiological functions

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

neuroscience, cardiovascular, stem cells, brain, spinal cord

Animal types	Life stages
Mice	neonate, juvenile, adult, embryo, pregnant, aged
Rats	juvenile, adult, aged, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The project aims to increase understanding of how the central nervous system is involved in the control of body functions, such as those controlling heart rate and blood pressure. In addition, it aims to understand how to new cells made in the adult central nervous system may interact with these circuits.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Modulating the activity of the nervous system by direct electrical or chemical intervention is increasingly under investigation for treatments of disorders such as hypertension, heart

failure and multiple sclerosis. However, knowledge of the neuronal circuitry underlying such modulation is far from complete and furthering such understanding is likely to contribute to development of more targeted and better treatments. Recent technological advances have improved the ability to gain this understanding and such a project is therefore timely.

What outputs do you think you will see at the end of this project?

The most likely outputs will be new information, which may lead to improved or new treatment for diseases that are influenced by nerve cell connections in in the spinal cord and brain, for example hypertension, heart failure, multiple sclerosis, spinal cord injury.

Who or what will benefit from these outputs, and how?

In the short term the most likely benefits will be to scientific researchers, who will use the outputs to build upon and further increase knowledge.

In the medium to longer term these advances in fundamental understanding may provide potential translational benefits. For example, understanding of central control of autonomic function gained from previous animal work has encouraged exploration of modulating the activity of the nervous system in humans through non-invasive electrical stimulation with the potential for treatment of diseases such as heart failure, hypertension and arthritis and indeed benefit some signs of aging. This fundamental work will examine the mechanisms underpinning such functions e.g. by identifying the anatomical and functional properties of constituent cells, how the cells are connected to each other and the functions of the networks. Understanding these will contribute to ongoing studies in humans.

How will you look to maximise the outputs of this work?

Collaboration is a mainstay in the laboratory - most of our publications have collaborative authors from other labs and indeed, from abroad. This increases the potential for the work to be seen by a larger audience and increase citations, disseminating knowledge effectively. Such collaboration will at least remain at the same level or indeed will likely increase.

We will also aim to present the findings at national and international conferences, where audiences often view outside of their close research area.

When appropriate we also produce press releases, which have led to widespread international media coverage and brought our research to the attention of the public. We will also continue with our public engagement where presentations based on our work have been made to events such as the national Pint of Science and events held by our University which are run annually, with around 1200 participants per day from the local community.

Species and numbers of animals expected to be used

- Mice: 8600
- Rats: 1500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use predominantly juvenile and adult mice and on some occasions, rats. Many of the fundamental processes that are controlled by neuronal circuits that we aim to understand are comparable to those in humans e.g. the baroreceptor reflex that controls blood pressure. In addition, increasing availability of transgenic mouse lines which enable specific cells to be identified and/or manipulated increase precision and specificity of studies in a manner that is not available in other species. To allow for development of circuitry we will mostly use adult animals, but in some cases they will require intervention at a younger age (from neonate) to promote expression of genes in specific cells.

Typically, what will be done to an animal used in your project?

In a typical experiment an animal will be terminally anaesthetised and either perfused with a fixative for anatomical studies, or will have tissue removed and sliced or en bloc for recording activity of cells ex vivo.

In some cases there will be a prior to inject substances will allow identification of specific cells in the nervous system.. This may require surgical intervention under anaesthesia, Animals will recover for up to 6 months, but more usually less than 4 weeks. Following this animals will be terminally anaesthetised and prepared as above.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals used only for anatomical studies or tissue slices do not recover from anaesthesia.

Post-surgical animals routinely receive suitable analgesia. Weight loss which is typically less than 10% is often seen on the first day following surgery and weight gain is within two days post surgery.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Preparing animals for anatomical studies or tissue slices is non-recovery. This is likely to account for 40% of the animals used in this project.

Animals which have had injections into the CNS may exhibit moderate severity (hunched posture, piloerection, transient anorexia, up to 10% weight loss) outcomes for a day following procedures. This is likely to account for 30% of the animals used.

Animals which have had intraperitoneal injections of substances typically exhibit mild severity (transient underactivity, piloerection), likely to account for approximately 30% of the project

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

The work will study how the CNS controls body functions, and at times how manipulating outflows of these circuits can influence progression of disease. These will necessarily involve placing tracers onto peripheral nerves to identify the cells or their origin, determine the effects of stimulating particular nerves or manipulate the signalling in the CNS to influence stem cells. Therefore, to provide physiological relevance whole animals are required. However, in vitro work will be undertaken where possible e.g. use of acutely prepared spinal cord slices for electrophysiology. In addition, use of cultured spinal cord slices will facilitate some experiments in vitro.. This allows for greater numbers of tests to be performed in vitro, reducing the number required in vivo.

Which non-animal alternatives did you consider for use in this project?

A possible longer term alternative is to use organoids derived from induced pluripotent stem cells or via cell reprogramming.

Why were they not suitable?

It is not yet possible to derive organoids that recapitulate the circuitry as they do not have the appropriate physiological inputs, such as fluctuations in blood pressure, changes in respiration etc. In addition, there is not enough information on circuitry to know if such organoids are faithful to the intact brainstem or spinal cord. Cell lines and cell cultures also do not have the necessary connections and appropriate circuitry.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to

design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been estimated with our experience of previous projects and accounting for breeding of transgenic animal lines. They allow for appropriate blinding of researchers as well as the use of either sex. We will conduct our experiments to be able to publish to the Arrive guidelines: https://www.nc3rs.org.uk/arrive- guidelines.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Most of our experiments do not have prior knowledge of effect size. When this can be estimated from our pilot studies we have used online resources such as the NC3Rs Experimental Design Assistant (https://eda.nc3rs.org.uk/experimental-design) to help calculate the number of animals.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We aim to breed as efficiently as possible. For example, cross breeding from homozygotes is preferred as this ensures that offspring carry the desired genes. Our institution has an online database that enable us to follow such breeding carefully.

If possible we conduct pilot studies on tissue slices, since many slices can be obtained from one animal and this enables pilot testing where several animals may otherwise be required.

The number of animals used will be optimised in several ways:

Tissue from the same animal will be used for different objectives where possible. This reduces the total number of animals that would otherwise be required if a single animal was used for each objective.

We have also developed a method of storing tissue for long term which also reduces animal use - this tissue is also available to other labs.

In addition, each experiment is designed to maximise the amount of information gleaned since they often combine different approaches to verify this information.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rats and mice will be used since they contain cell types and assemblies of cells that are similar to those known in humans as far as can be told so far. Transgenic animals will be used when they make identifying and/or manipulating specific cell types possible with fluorescent markers, helping to minimise numbers used.

Suffering of animals will be minimised as the majority of the procedures performed will have a minor severity where the animal is killed by anaesthesia prior to organ removal. In some cases experiments will require recovery from anaesthesia following surgical procedures. Post-operative analgesia will be applied in these cases to limit suffering of the animals.

Why can't you use animals that are less sentient?

We cannot use more immature animals as we are studying the control of the autonomic nervous system and this is not properly developed until the adult stages.

Less sentient species (such as flies and worms) do not have the same autonomic nervous system control.

We will use terminally anaesthetised animals in many of our experiments, but some will require surgical intervention with recovery to allow expression of markers or functional controllers in sets of cells

beforehand or to mark the terminations of specific nerves. There is no current approach to do this without surgery.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Suffering of animals will be minimised as the majority of the procedures performed will have a minor severity where the animal is killed by anaesthesia prior to organ removal. In some cases experiments will require recovery from anaesthesia following surgical procedures. Post-operative analgesia will be applied in these cases to limit suffering of the animals. Examples of refinements that we have made include, peri--operative administration of warm saline to ensure that the animals remain hydrated, adding food mashed with water to the cages after surgery so that there is easy access for eating and maintaining hydration post-surgery, housing with other mice wherever possible (which is usually the case).

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The NC3Rs have published best practice guidance: (Prescott MJ, Lidster K. Improving quality of science through better animal welfare: the NC3Rs strategy. Lab Anim (NY). 2017 Mar 22;46(4):152-156. doi: 10.1038/laban.1217. PMID: 28328893) and this and subequent publications will be referred to. We attend webinars from Nc3R like "the best practice in experimental design": https://www.nc3rs.org.uk/events/webinar-best-practice-experimental-design.

In addition, the ARRIVE 2.0 guidelines (https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.3000410) will be referred to assist with good design as well as reporting.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I, and my lab members, take part in CPD regularly. This includes courses run internally as well as reading appropriate literature including N3CR resources such as web sites and the newsletters we receive. In some conferences, there are posters and sessions dedicated to the 3Rs. We discuss our experiments with other researchers, NACWO and the vet, implementing appropriate changes as we go through projects.

187. Neuronal circuits regulating metabolic and cardiovascular health

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Obesity, Cardiovascular Disease, Metabolic Disease, Neuroscience, Stress

Animal types	Life stages
Mice	juvenile, adult, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Obesity, diabetes and cardiovascular disease are increasingly prevalent conditions in our society, often exacerbated by mood disorders, including stress and anxiety. This project aims to provide a precise understanding of the brain circuitry regulating and linking these processes with the ultimate goal of devising better treatments to treat and to prevent these diseases.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Obesity, diabetes and cardiovascular disease are a major health burden for the individual suffering from it as well as a financial and social burden for our society as a whole. These diseases are interlinked, and it is becoming increasingly clear that our nervous system, particularly the autonomic nervous system, that is not under cognitive control, regulates the variables such as energy balance, blood glucose levels or blood pressure and heart rate which go wrong in these diseases.

The hormone GLP-1 is perhaps the single most promising therapeutic target for the treatment of these disorders with several GLP-1-based therapies already approved for the treatment of obesity and diabetes. Our work (and that of other research laboratories) over the past decade focussing on GLP-1 has demonstrated that the robust appetite-suppressive effects of GLP-1 are mediated by the brain and that GLP-1 produced by the gut and GLP-1 produced by the brain work independently to decrease food intake. This highlights an untapped potential for brain GLP-1 in the treatment of obesity, but the neural circuits underlying the effects of brain-produced GLP-1 on feeding are largely unknown. In addition, recent work by our group and others suggests that GLP-1 released within the brain may not simply suppress appetite but also drives responses to stress, including increased heart rate, stress hormone release, and anxiety-like behaviours. Further studies have highlighted that GLP-1 within the brain reduces the reward value of food (and also of substances of abuse) and furthermore reduced loss of neurons in neurodegenerative diseases such as Alzheimer's dementia has been ascribed to actions of GLP-1 on the brain. The neuronal circuitry underlying the regulation of these various responses to GLP-1 is currently largely unknown, but this knowledge is essential to tailor clinical treatment strategies, primarily for obesity, but potentially also for substance abuse and neuroprotection linked to stroke and neurodegenerative disease, and keep unwanted side effects at a minimum.

Research covered under this project will provide a detailed understanding of the neuronal circuitry and functionality underlying both the effects of systemically administered GLP-1 receptor agonists, and those triggering the release of native GLP-1 within the brain. This is expected to lead to the development of improved strategies for weight control, by refinement of current approaches for blanket activation of GLP-1 signalling pathways to those allowing targeted activation of functional subcircuits within the brain. This level of insight can only be achieved by utilising the latest developments in genetic manipulation available in animal models, selective viral gene transfer and imaging of neuronal activity in living brain tissue. Currently this is only possible in specific mouse strains which provide us with unique opportunities of gaining this level of functional insight into a mammalian brain.

What outputs do you think you will see at the end of this project?

This project is likely to increase understanding of how autonomic nervous system function in general, and GLP-1 in particular, affect food intake, bodyweight, blood sugar and cardiovascular control. All results will be published in scientific journals, and thus be publicly available. Depending on its results, this programme of work might influence policy on the use of GLP-1 analogues, such as liraglutide, as anti-diabetic and anti-obesity drug.

It will also generate data to establish the contribution of autonomic nervous system dysregulation to diabetes and obesity and thus open new treatment avenues.

Who or what will benefit from these outputs, and how?

The primary output from this project will be a stream of publications throughout the duration of this project. This will incrementally add more information to our knowledge of the neuronal circuits regulating energy balance. Thus, the earliest beneficiary will be the scientific community, as our studies will inform future studies of ourselves and other laboratories worldwide. The second level of impact is likely to be the potential impact on drug development projects by the pharmaceutical industry and on the way how clinicians think about the diseases and treatment strategies. The final long-term benefit for patients would be realised when/if our results lead to new/altered clinical treatment regimes.

How will you look to maximise the outputs of this work?

We are looking at maximising both quality and quantity of output as well as impact of this work. The former we achieve by ensuring we always use the most appropriate experimental strategies that provide clear, unequivocal results. This is achieved by keeping informed about the latest developments in the field and collaborating with the best international laboratories. We are able to do this, because our laboratory has an excellent international reputation in our field and consequently the top laboratories are happy to work with us. We also constantly disseminate our results to the scientific community via conference presentations, invited seminars and publications (both primary and review articles). Additionally, we talk directly to industry stakeholders and we have recently started a dialogue with a biotech company aiming at developing a novel small molecule GLP-1 receptor agonist with bespoke properties in terms of brain penetration.

Species and numbers of animals expected to be used

- Mice: A maximum number of 5500 mice will be bred and used under this licence.

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This work involves evaluation of the functional interaction between various organ systems (e.g. brain, digestive tract, heart). Such complex interaction cannot be reproduced with cell cultures and thus has to be performed using live subjects. Since the organ systems should be as similar as possible to humans, these experiments have to be performed on mammals. Additionally, the functional manipulation of the specific cellpopulations inside the brain that are the subject of this project are only possible in transgenic animals.

Consequently, only a select few transgenic mouse strains are able to support this project and provide the precision in the manipulation required to succeed.

Finally, type 2 diabetes mellitus, obesity and cardiovascular disease are conditions that mainly afflict the adult (and aged) population. Consequently, wherever possible the experiments under this licence will be performed on adult (and sometimes aged) mice, and manipulation of the functionality will be performed at this developmental stage by employing viral gene transfer, rather than using transgenic mouse strains that e.g. lack specific genes from conception and thus can be prone to developmental compensation or exacerbation. In contrast, our transgenic strains only enable the viral manipulation in the adult animal, thus avoiding any potential effects during development.

Typically, what will be done to an animal used in your project?

This project uses transgenic mice which enable us to both visualise as well as manipulate the neuronal circuitry involved in mediating GLP-1 effects in the living organism. Thus a large proportion of the animals utilised will be needed as tissue donors for subsequent analysis of functionality and anatomical organisation of this circuitry in the isolated tissue. These animals will simply be euthanised under terminal anaesthesia. More complex experiments aim at understanding the consequences of manipulation of the neural circuits under investigation in the living animal. For those experiments transgenic mice will undergo stereotaxic injection of viral constructs into the brain followed by behavioural tests a couple of weeks later to assess the impact of interfering with specific brain circuitry on e.g. food intake, stress resistance, cardiovascular health, etc.

What are the expected impacts and/or adverse effects for the animals during your project?

The functional consequences of the viral manipulation of the neural circuitry under investigation are expected to be benign. Work under our previous licence has shown less adverse effects (such as nausea) than what is described by patients who take GLP-1 based drugs as anti-diabetes medication. Similarly, the transgenic mouse strains that we use do not show any adverse effect in themselves.

The main adverse effects that we expect are transient, and result from the surgery itself. Thus, the mice tend to suffer from post-operative pain for up to 24h after virus injection through the skull, and for up to 48h after injection into brainstem or spinal cord, because this involved some damage to muscle tissue in neck or back. Similar recovery times are seen after implantation of cannulae into the brain parenchyma or ventricles, or implantation of telemetry devices to monitor blood pressure and heart rate. After this time no difference in behaviour is observed with post-surgical mice compared to untreated littermates.

Additionally, when undergoing specific behavioural tests or measurement of food intake, the animals will experience stress in form of handling, injection, exposure to novel environments and novel food. Where diets are changes longer term to highly palatable or

high fat diet, this generates an obesogenic and consequently diabetogenic environment. Thus, they are monitored for any signs of overt diabetes or other adverse effects of excessive weight gain. All of these protocol steps are classified as mild.

Finally, a subset of mice will be exposed to acute or chronic stress in established rodent stress models. This is expected (and indeed intended) to elicit a level of distress for a period of time, usually limited to hours in the case of acute stress and a few weeks in the case of chronic stress. These types of stressful experiences are essential to understanding the neural circuits mediating harmful effects of stress. The mice are carefully monitored throughout these procedures.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The vast majority of animals used under this project are experiencing 'mild' severity. This is the case because breeding of transgenic strains is per definition 'mild'. The only invasive procedure is ear notching for genotyping, and otherwise just handling of the mice for husbandry. The severity for those animals that are simply tissue donors or undergo procedures under terminal anaesthesia is 'subthreshold', as they only experience induction of anaesthesia, and for those animals undergoing recovery surgery it is 'moderate', because of surgical trauma and the healing process. None of the behavioural and other tests the animals are undergoing prior and or after surgery produce any significant or lasting discomfort that would require classification as 'moderate'.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This work involves evaluation of the functional interaction between various organ systems (e.g. brain, digestive tract, heart). Such complex interaction cannot be reproduced with cell cultures and thus has to be performed using live subjects. Additionally, the precision of the manipulation of brain cells can currently only be achieved in transgenic mice.

Which non-animal alternatives did you consider for use in this project?

For the reasons outlined in the paragraph above, there are no non-animal alternatives applicable for this project. All work under this licence will be performed using the least

sentient preparation possible. This means that for those parts of the project that involve analysis of brain anatomy only, or functional responses that can be elicited directly on the neurons under investigation and are measurable as direct cellular responses, animals will only be used as tissue donors. Even for this part of the project the use of cell lines is not applicable, because results depend on the expression of the specific set of receptors by the specific set of neurons in the mature brain.

Why were they not suitable?

Neurons are not proliferating, thus there cannot be a cell line replicating the properties of these specific neurons, and primary cell culture would still require the use of donor animals, with the caveat that even primary cultures tend to change the expression of receptor proteins and have to be obtained from immature animals, which puts the phenotype of the actual cells investigated in such a preparation into question.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals used in this project over the duration of five years can only be a rough estimate and is designed to give a maximal number that will definitely not be exceeded. This is because the majority of experiments built upon each other, and thus it is extremely difficult to accurately calculate the exact number of animals needed. This is very different for each individual experiment, which are powered based on the magnitude of the effects observed previously with similar genetic models and experimental paradigms. Sex-balanced cohorts of 8 animals will typically allow us to resolve significant changes in food intake when manipulating the GLP-1 system.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

While our experiments will generate valuable data resulting from novel manipulations of neuronal populations, the majority of behavioural analyses used to determine the effects of these manipulations have been optimised and standardised in the lab over many years. They have therefore undergone considerable empirical refinement, and been confirmed as appropriately powered and designed with G*Power 3.1.9 and EDA. Two examples of protocol optimisations which reduce animal numbers are our approach to experimental controls when using new chemogenetic effectors, and our treatment of sex as a biological variable (SABV). When new chemogenetic effectors are used, behavioural control experiments will be conducted using 2x2 mixed model designs, whereby mice are

transduced with either the effector or a Cre-dependent fluorescent reporter control virus. The effect of the activating ligand (CNO) will then be quantified in both groups. A lack of difference between the virus groups in the control condition will confirm the effector has no constitutive effect. Observation that the effect in the active condition (CNO) is restricted to the group transduced with the effector will confirm that the effect is not driven by off-target actions of CNO. To balance experimental rigour with the need to reduce animal numbers and refine experimental designs, once the functional specificity of each new effector has been validated, subsequent experiments using the same approach will be conducted using 1x2 within-subjects designs in chemogenetic effector-expressing mice only. This will increase statistical power by minimising the influence of inter-animal variability, and substantially reduce the total number of animals required for the project. In our experiments to date, we rarely observe significant effects of sex on our dependent variables. All behavioural experiments are nevertheless designed to balance consideration of SABV with the need to reduce animal numbers. Therefore, sex-balanced experimental cohorts are used of sizes which are appropriately powered to detect effects which are present in both sexes, and sufficient to provide a reasonable indication when they are not. In the latter case, experiments are repeated in a second sex-balanced cohort, such that results can be appropriately pooled and analysed for sex effects, without the confound of batch effects.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Since all genotypes are useful and we use both males and females, we use virtually all mice produced through breeding. In addition, individual animals will be subjected to more than one test each and after the in vivo program their tissue will be used for further in vitro analysis, thus reducing the overall number of animals required.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

This project requires the use of mammals, because the control of food intake and responses to stress by the brain are too different from man in other vertebrates, or invertebrates. Since these functions are fulfilled mainly by ‘lower’ parts of the brain rather than cerebral cortex, rodents are seen as adequate model systems. Mouse and rat can be bred and maintained in a laboratory setting without too much impact on the welfare of the

individual animal. In this case we have to use mice for our work, because the genetic modifications required for this project are only available in mice. Individual animals will receive viral gene transfer that will incorporate a ‘switch’ into their GLP-1 producing cells, cells upstream of these, GLP-1 responsive cells, or into their parasympathetic vagal neurons, that allows us to ‘switch on or off’ these cells at will. The effect of such manipulation on food intake, body weight, blood glucose control and blood pressure or heart rate will then be measured. These measurements will be performed as much as possible with implanted devices that transmit data remotely (telemetry) which reduces handling, and therefore stress, for the animals, leading to better data with smaller variations, and thus might also lead to a reduction of the number of animals needed.

Why can’t you use animals that are less sentient?

The GLP-1 system that is the main subject of our studies appear to be remarkably conserved throughout vertebrate species from fish to man. Thus, some of the anatomical and descriptive experiments could potentially be performed in other vertebrate species than mouse. However, this project relies on our ability to functionally manipulate those neurons producing GLP-1 or those neurons responding to GLP-1. Currently this is only possible using transgenic mice and not in either higher or lower vertebrates. Outside of vertebrates we would expect to find somewhat equivalent control circuits for food intake, stress, etc, but the applicability of the findings to humans would be a lot more questionable.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Due to the relatively large number of surgeries required to generate experimental cohorts, we consider optimisation of our protocols for surgical anaesthesia/analgesia and post-operative care of particular importance, and hence regularly review these in conjunction with the NACWO and/or NVS to ensure any updates to best practice are swiftly implemented. For example, we have recently refined our post- operative care protocol to provide a warmed home-cage recovery environment for mice for the first night after surgery, and post-operative analgesia in an edible formulation that ensures consistent doses without the need to perform injections. We have also refined our protocol for automated food intake monitoring by upgrading to the FED3 system, which allows pre-programmed timed feeding schedules to reduce the stress of manual food removal and return during fast/refeed protocols.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Our experimental design and procedural practice will be informed by best practice guidance published from a variety of sources. In particular, we will utilise the comprehensive list of recommended resources curated by the UCL BSU Named Information Officers, including information from the Home Office, NC3Rs, Laboratory

Animal Science Association (LASA), and Federation of European Laboratory Animal Science Associations (FELASA).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Our Biological Services Unit (BSU) is very proactive in disseminating the latest advances and best practice guidance regarding the 3Rs to all users of their facilities. In addition to this excellent source of information, we keep ourselves regularly appraised of general and field-specific advances via social media (e.g. by following The National Centre for the 3 Rs twitter account and blog). Opportunities to implement new 3Rs advances will be taken as soon as possible, via formal and/or ad hoc online and in-person BSU training courses, and refinement of our specific methodologies.

188. Analysis and therapy of cerebellar disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

cerebellum, ataxia, neurodegeneration, Purkinje cells, therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim of this project is to better understand the disease-causing mechanisms underlying cerebellar disorders with a focus on cerebellar ataxia and to develop effective therapies.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

The cerebellar ataxias are a complex group of brain disorders. Patients suffering from ataxia have problems with their movement, balance and speech, and sometimes develop cognitive disabilities. These clinical symptoms strongly impair patients' quality of life, and

many ataxias lead to premature death. The many genes implicated in cerebellar ataxia together with the clinical complexity present a challenge in our understanding of the underlying disease-causing mechanisms and has hampered the development of effective therapeutics. As a result, to date no effective treatments exist to slow or halt cerebellar ataxia. Current clinical care remains largely symptomatic and has only modest benefits.

This project will provide a better understanding of the disease mechanisms in cerebellar ataxia and identify novel clinical intervention points and potential therapies. This will improve the quality of life for ataxia patients and their families.

What outputs do you think you will see at the end of this project?

This project will generate new scientific knowledge about the disease mechanisms and treatment options for cerebellar ataxia. We will communicate our findings through open-access, peer-reviewed publications and accompanying press releases. Research findings will also be shared through presentations at national and international conferences and invited seminars. In addition to publications and presentations, we expect the development of new scientific collaborations and funding applications as a direct result of this project. Finally, this project aims to identify candidate therapeutic compounds that will lead to novel IP and the future commercialisation of therapeutics for the treatment of cerebellar ataxia.

Who or what will benefit from these outputs, and how?

In the short-term, the knowledge generated in this project will provide a better understanding of cerebellar ataxia and will benefit the scientific community. In addition to basic scientists, this work will also be important for clinicians and clinical geneticists in cerebellar ataxia as it will provide important insights into human genetic variants in cerebellar ataxia and thus help to improve molecular diagnoses for ataxia patients. Ultimately, the project aims to identify and test novel therapeutics for cerebellar ataxia. This will lead to the development of an effective treatment for this group of disorders and improve the quality of life for patients and their families. The latter benefit is unlikely to be fully realised after five years. However, we hope to have identified at least one compound that could be taken further into clinical studies.

How will you look to maximise the outputs of this work?

We regard the dissemination of our research findings as a high priority and will communicate our findings through open-access, peer-reviewed publications, presentations at conferences, and invited seminars. Our developed methods, materials, and models will be shared with the scientific community. We have established collaborations with clinical geneticists and neurologists both in the UK and internationally and we will share our findings with them so that our results can have an immediate impact in the clinic. We will also actively engage with patient organisations to share our research findings. Finally, we are committed to public engagement of science and will widely communicate our research activities through participation in outreach events such as science festivals, public talks and school visits.

Species and numbers of animals expected to be used

- Mice: 33,000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice will be used for this project because their cerebellum is very similar to the human cerebellum in both its structure and physiology. This allows the study of specific structures and cell types that are specifically affected in cerebellar disorders and that are not present in lower species. The genetic standardization and toolkit available for mice also make this animal an ideal model to study the functional effects of genetic mutations. Moreover, the mouse is well-suited for behavioural testing and pharmacological interventions that are directly applicable to humans.

Cerebellar ataxias are degenerative brain disorders with an onset of disease throughout lifetime. We therefore need to use adult and ageing mice for our project. We will also study the developing cerebellum in mouse embryos and juvenile mice as many of the cerebellar disorders that we investigate cause early changes during brain development.

Typically, what will be done to an animal used in your project?

Typically, a mouse in this project will undergo a series of non-invasive, behavioural tests at different times during its lifetime, from neonatal stages through to ageing, to assess motor and cognitive function. The order and timing of the behavioural tests used in this project will be planned to cause the least stress to the animals while still providing the most biologically relevant data. Generally, a testing regime over a number of days will begin with the least stressful first and will have the most stressful test at the end of the protocol. No more than ten non-invasive tests will be carried out per testing period with a maximum of six testing periods over the course of the animal's lifetime.

A mouse might be given a compound for up to three months to improve motor behaviour and to test potential therapeutic approaches for cerebellar ataxia. We will aim to administer compounds orally as this is the most refined route, but occasionally compounds might need to be administered via injection.

At the end of these studies, the mouse will be humanely killed to collect tissue for pathological and biochemical studies.

What are the expected impacts and/or adverse effects for the animals during your project?

The most likely adverse effects will come from the genetic modification of the used mutant mice. The project uses mouse models of cerebellar disease that display features similar to human patients, i.e., progressive dysfunction and loss of cerebellar nerve cells that will result in movement problems.

Typical symptoms might include ataxia, tremor, weight loss, and altered activity. These

symptoms are expected to start within the first three months and will progressively worsen over the animal's lifetime. Mice will be monitored and scored to assess welfare and will be treated with nutritional support where appropriate.

Animals may undergo procedures such as repeated motor function tests and injection, which are minimally invasive, and no significant adverse effects are expected.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Sub-threshold (10% of animals)
- Mild (30% of animals)
- Moderate (60% of animals)
-

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Central to this project is the characterisation and treatment of human brain disorders. This requires the study of the brain in a living animal. Moreover, the studied diseases manifest as deficits in complex behaviours, which cannot be examined in cell or other models. Also, it is not possible to mimic the action of a drug on the brain from cell lines alone as the drugs for treatment will have to be delivered by the blood stream. Regulatory authorities also demand that we have pre-clinical data in mice before going to human clinical trials.

Which non-animal alternatives did you consider for use in this project?

Post-mortem brain material could be used to gain insight into pathological changes in the human brain; for example, to determine which cell types are lost and in which regions of the patient cerebellum.

Tissue culture models are used where appropriate; for example, to investigate cellular pathology and to see if any therapeutic compounds can correct the disease at the level of the single cell.

Moreover, our group has developed a reliable method to generate cerebellar nerve cells from human induced pluripotent stem cells (hiPSCs). These can be derived from control and patient fibroblasts and offer the possibility to investigate pathology in disease-relevant living human nerve cells.

Why were they not suitable?

Post-mortem brain material is scarce and not available for all genetic subtypes of cerebellar ataxia. Moreover, human post-mortem tissue is usually obtained at an advanced and/or end-stage of the disease and therefore offers only limited insight into early pathological changes.

While hiPSC-based models are an exciting new technology, the generated neurons are relatively immature and do not yet recapitulate changes that occur in the adult brain.

Importantly, studies of brain behaviour and function are currently not possible in vitro as cell-based systems do not replicate the complex in vivo interactions of the brain. Also, no cell model can offer an alternative to behavioural studies in an intact animal.

Finally, although the screening of therapeutic compounds is initially performed in cell models, it is still necessary to determine whether these treatments have any therapeutic benefit in intact living animals prior to translational studies in patients. This is also a regulatory requirement.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used in this license has been arrived at through statistical advice and from our own personal experience of similar work carried out previously. The majority of animals will be used for breeding.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Experiments have been designed using the online NC3R's Experimental Design Assistant and in consultation with experts in experimental design.

In our experimental design, we will carefully consider minimal group sizes, number of groups to be studied, using one or both sexes of animals as appropriate. We will consider factorial design of the experiments to increase the amount of information gained. For behavioural testing, we will consider controlled multiple testing, which facilitates obtaining the maximal amount of information from an individual animal. Where practical, we will use computer-generated randomisation protocols to randomise experimental groups. Experiments as well data analysis will be performed blinded to minimise bias introduced by the experimenter. For behavioural experiments, counterbalancing of the experimental design is important to ensure that results are not attributed to technical variation, for example a specific apparatus or other variables (e.g., starting position in a test or order in which stimuli are presented).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The use of in vitro methods, for example in cell models prior to the animal experiments, will reduce the number of animals required to attain statistical significance.

We will set up the minimum number of matings required to ensure adequate numbers of the desired genotypes are produced. We will maintain a minimum colony size for each mouse model by using data generated by our mouse database. We will constantly analyse and review our data to ensure that the lowest possible number of animals per group are used. We will continue to ask for advice from experts in experimental design and others in the field whenever required to maintain a rigorous approach to experimental planning and data analysis.

As group sizes needed for any study are based on the effect size being measured, we will use small- scale pilot studies to inform us on effect size so that the main study can be designed to be adequately powered and avoid over-use of animals.

A wide selection of tissue samples will be taken from each animal used in a study to try to cover every eventuality with regards to any potential additional analyses which we may want to perform and prevent having to use more mice in a repeat experiment. This ensures maximum use of every animal that we work with. Samples are put in long-term storage enabling us to access them if and when required at any time in the future.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mouse models of human cerebellar disease will be employed as these are the lowest vertebrate model that we can use to achieve the purpose of this project with enough aspects of genetics, anatomy, physiology and behaviour shared with humans to generate biologically relevant data that can ultimately be used to develop novel therapies.

We will use the most refined methods according to published literature and in consultation with experts in the field. Regular examination of the animals by trained staff and experienced technicians will ensure that steps are taken to minimise any distress or discomfort to the animals. Veterinary advice will always be sought where and when necessary.

Why can't you use animals that are less sentient?

The aim of this project is to employ accurate models of human cerebellar disorders to better understand these diseases and develop novel therapies. This aim can only be achieved in animals that mimic the human condition and show clinically relevant phenotypes such as progressive loss of neurons in the adult brain and behavioural impairments.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have photo and video material of the ataxia models available that we will share with the team to provide detailed information about the expected disease phenotypes. Animals will be monitored appropriately dependent on their phenotype or known adverse effects and stage in a specific protocol. We will also continue to assess the disease endpoints used to minimise suffering. We will keep abreast of the literature and adopt more refined disease models, e.g., inducible and/or conditional models, when these become available.

Care and husbandry refinements will be made including the use of mash and jelly in the diet of the mice to ensure they can feed even if mobility is reduced. This is expected to result in improved health and extended lifespan of the animals.

For behavioural testing, we will continue to refine the protocols with respect to both scientific merit and animal welfare at all times by review of the literature and communication within the establishment and other experts in the field. The order and timing of the behavioural experiments will be planned to cause the least stress to the animals while still providing the most biologically relevant data. Generally, a testing regime over a number of days will begin with the least stressful test first and with the most stressful test at the end of the protocol.

For the testing of potential therapeutic compounds, we will base dosages on published work with close consultation with other experienced research groups so that the most refined approach is used. When a novel compound is used, information from other experimental data such as in vitro studies will be used to predict toxicity and estimate predictable doses; a pilot study will also be carried out initially.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will follow best practice guidance according to the Handbook of Laboratory Animal Management and Welfare by Wolfensohn and Lloyd, as well as published literature on most refined methods in the field. For behavioural protocols, we will use published and regularly updated protocols where available, for example from the International Mouse Phenotyping Consortium (IMPC). We will also consult the publications from LASA (Laboratory Animal Science Association) and AHWLA (Assessing the Health and Welfare of Laboratory Animals) for useful guiding principles. We will conduct and report experiments according to the published ARRIVE guidelines.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Advances in the 3Rs are regularly communicated by the establishment and its dedicated 3Rs information officer and local 3Rs regional programme manager. We will attend an annual 3Rs Research Day, where researchers inform the community about advances and best practices in animal research with respect to the 3Rs. Advances in the 3Rs that are relevant to this project will also be discussed and implemented in close consultation with the named people.

189. Vascular protective genes in angiogenesis

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Pregnancy, Angiogenesis, Preeclampsia, Cardiovascular, Antioxidant genes

Animal types	Life stages
Mice	pregnant, adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The purpose of this project is to investigate whether certain natural protective factors can reduce the severity of two medical conditions: preeclampsia (PE) and cardiovascular disease. The research team aims to understand more about the underlying mechanisms involved in these disorders. To achieve this, they will be using established models of PE and atherosclerosis in mice to study the role of certain genes, such as heme oxygenase, in these conditions. The study has two main parts: i) Testing the role of genes that have been shown to help alleviate the symptoms of PE and cardiovascular disease. ii) Utilizing two new models of PE to identify important genes in the development of this condition. The first model is a surgical model that mimics PE, but without any genetic manipulation. The

second model involves manipulating specific placental cells to understand the role of different genes in PE.

In short, the project aims to further our understanding of the factors involved in preeclampsia and cardiovascular disease and how they can be mitigated.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Disorders related to the placenta during pregnancy can result in a range of complications for both the mother and her baby, including preeclampsia (PE) and fetal growth restriction (FGR). PE is characterized by high blood pressure and poor kidney function and FGR affects approximately 10% of all pregnancies. These conditions also increase the risk of heart disease and other adult diseases like diabetes for both the mother and baby in the future. Despite the importance of PE and FGR, there are currently no therapies to treat them. This is due to a lack of understanding about why some placentas don't function properly during pregnancy and the reluctance of drug companies to invest in treatments for use during pregnancy due to the potential risks to both the mother and baby. To do this, it is important to test existing and new drugs, especially those that are considered safe during pregnancy, in animal models of PE and FGR to see if they have the potential to be used in human patients. By improving our understanding of why some placentas don't function properly and testing new treatments, we can work towards improving outcomes for mothers and babies affected by PE and FGR.

What outputs do you think you will see at the end of this project?

The aim of this research program is to improve our understanding of the processes involved in blood vessel growth, known as angiogenesis. Angiogenesis is critical for many physiological and pathological conditions, including peripheral vascular diseases, heart attacks, strokes, wounds, tumors, and retinal disorders. The ability to both promote and inhibit blood vessel growth is crucial for addressing these conditions. To date, most of the research on angiogenesis has been done in laboratory conditions using cells grown in a dish. However, these conditions are not representative of the complexity and variability of angiogenesis in living organisms. Therefore, this research program aims to study angiogenesis in live animals to gain a more accurate understanding of the mechanisms involved.

The primary focus of this licence is to determine the role of specific genes in angiogenesis, and to complement the in vitro studies on endothelial cells that have indicated a potential role for these genes in supporting capillary growth. Additionally, the long-term objectives of the research include identifying new targets for the treatment of vascular disorders such as

preeclampsia and understanding the molecular basis of blood vessel growth and remodeling.

Our research is unique, both nationally and internationally, in its studies on angiogenesis in vivo. Such studies are crucial for advancing our understanding of the mechanisms involved in angiogenesis and for translating this knowledge into the clinical setting. The results of this research have the potential to provide new insights into the treatment of various vascular disorders and could lead to the development of more effective therapies

Who or what will benefit from these outputs, and how?

In short, this project will provide valuable information and insights into the causes of poor pregnancy outcomes and cardiovascular diseases. The findings from this study will be of benefit to scientists, clinicians, and medical professionals in the fields of reproductive health and vascular diseases. Additionally, this research will directly benefit patients, particularly women who are suffering from disorders of pregnancy. In the long term, the results from this project will contribute to the development of new and effective treatments for these conditions.

How will you look to maximize the outputs of this work?

The results of the project will be shared with the scientific community through publication in scientific journals and presentations at scientific conferences. This will be done with the goal of reaching both specialists in the reproductive and cardiovascular fields, as well as a broader audience through multidisciplinary journals. The team will also continue to build new collaborations and seek out new knowledge to improve understanding of the causes of poor placental function and to identify potential new treatments for vascular diseases. The researchers will also be in regular communication with their patient information panel to ensure that the research is being done with the patients' needs in mind and will work to educate the public about their findings through community events.

Species and numbers of animals expected to be used

- Mice: 6000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The use of mice to study the role of vascular genes in normal angiogenesis and in pregnancy is a well-established and highly valuable approach in scientific research. The development of transgenic mouse models that are susceptible to changes in vascular

factors that allow for the investigation of specific mediators and pathways has greatly increased the use of mice in this field. These models provide the best and most versatile models of vascular disease. Additionally, the use of mice has been further improved through the development of techniques such as transient knockdown strains, surgical procedures, and high-resolution imaging.

However, it is important to note that not all results from murine models can accurately predict the effects of treatments in humans. Therefore, it is often necessary to confirm positive findings in additional models. In this project, three novel models will be used to investigate complications during pregnancy and related vascular disorders. This multi-model approach will provide a more comprehensive understanding of the underlying mechanisms involved.

The main aim of this project is to understand the role of vascular genes in controlling in vivo angiogenesis. This will be achieved through surgical manipulation of the vascular system in mice, combined with a high-fat diet in some instances. The process of remodeling will also be manipulated in some cases, by altering maternal blood pressure, altering vascular factor concentrations of proteins in circulation, or administering pharmacological agents. The use of genetically modified mice will help to investigate the role of specific molecular pathways in angiogenesis. At the end of the experiments, the animals will be sacrificed to obtain tissue samples for laboratory analysis. The results will be compared to laboratory experiments using cell cultures or placental samples from humans. To imitate preeclampsia in mice, adenoviral gene transfer will be used, and the reduced uterine perfusion pressure (RUPP) model will also be utilized. The RUPP model mimics the hypertension, immune system abnormalities, systemic and renal vasoconstriction, and oxidative stress in the mother and intrauterine growth restriction in the offspring. This allows for investigation of the origins of preeclampsia phenotype without the influence of a specific gene.

Typically, what will be done to an animal used in your project?

The experiments will aim to study the effect of different factors on vascular remodeling and how they contribute to pregnancy complications and related disorders. This will be done by inducing vascular remodeling through surgical manipulation of the vascular system and, in some cases, by combining it with a high-fat diet in mice. At the end of the experiments, the animals will be sacrificed, and their tissues will be collected for laboratory analysis.

Additionally, the use of genetically modified rodents will allow for investigation of the role of specific molecular pathways in these processes. The experiments will be designed and conducted based on previous research and findings in the field and will also be supplemented by laboratory experiments using cell cultures or biological samples from humans. This project will provide insight into the molecular mechanisms underlying angiogenesis and vascular disorders and contribute to the development of new treatments for related health issues.

All surgical procedures and experimental protocols will be reviewed and approved by an institutional ethics committee. The use of animal models is necessary to advance our understanding of the role of angiogenic and antioxidant genes in placental function and to identify new treatment options for vascular diseases. However, every effort will be made to minimize animal suffering and use the minimum number of animals required to obtain valid scientific results.

a) Induction of preeclampsia – i) Maternal Vascular Disease - In non-pregnant mice, we will investigate the underlying molecular mechanisms of maternal vascular disease using a high-fat diet-induced model of oxidative stress and inflammation. This will help us understand the effect of oxidative stress and inflammation on the maternal vasculature and the resulting long-term endothelial cell dysfunction. ii) Atherosclerosis - In non-pregnant mice, we will use the high-fat diet-induced model of atherosclerosis to investigate the underlying molecular mechanisms of this disease. We will examine the role of specific mediators and pathways in the development of atherosclerosis and the resulting cardiovascular complications. iii) Angiogenesis - In non-pregnant mice, we will investigate the molecular mechanisms underlying angiogenesis using various models such as the inhibition of angiogenic factors and the stimulation of angiogenic pathways. This will allow us to understand the role of angiogenic factors in the development of various diseases, including cardiovascular disease and cancer. All the mice used in this project will be maintained with the use of appropriate anesthetics, analgesics, and euthanasia methods to minimize animal suffering.

b) To replicate these diseases in rodents, adenoviral gene transfer and reduced uterine perfusion pressure (RUPP) models will be used. In the adenoviral gene transfer model, antiangiogenic genes will be transferred, leading to angiogenic imbalances and clinical symptoms of preeclampsia. The RUPP model simulates hypertension, immune system abnormalities, systemic and renal vasoconstriction, oxidative stress, and intrauterine growth restriction in the mother and offspring. This allows for investigation of the origins of preeclampsia without the influence of specific genes through their loss or overexpression. Preeclampsia will typically be induced in mid-gestation pregnant mice and the experiment will be terminated near term. The users has gained experience and confidence with this procedure over the last five years, with no adverse outcomes reported during experimentation.

c) Modulation of atherosclerosis – The mice used in this project will be given a high-fat diet to induce atherosclerosis, which is the build-up of plaque in the arteries. This will be done by modifying their diet for 4-12 weeks. The dietary modification will take place in both wild-type and genetically altered mice. The goal of this experiment is to study the biological processes and interactions that underlie the development of atherosclerosis and to examine how genetic alterations affect this process. The mice will be allowed to have unlimited access to the modified diet during the experiment.

d) Induction of angiogenesis – the use of animal models in the study of angiogenesis aims to provide a deeper understanding of the mechanisms underlying the regulation of

new blood vessel formation. Three complementary models will be used: physiological angiogenesis, corneal micro-pocket assay, and pathological angiogenesis. These models will help to clarify the mechanisms of regulation and allow the evaluation of potential therapeutic options. The experiments will last up to 14 days, with monitoring of mice and assessment of angiogenesis using relevant imaging techniques. The study may include species other than mice, such as transgenic animals, to improve the relevance of the results. These parameters may include changes in blood vessel size, structure, function, and permeability. Non-invasive imaging techniques such as ultrasound scans can be used to visualize and quantify these changes. Additionally, histological analysis of blood vessel tissue can provide detailed information on changes in the tissue architecture and cellular composition. Other measurements that may be made include changes in blood flow and pressure, as well as changes in the expression of key signaling molecules and growth factors involved in vessel remodeling. By combining these different measurements, researchers can gain a comprehensive understanding of the underlying mechanisms of vessel remodeling and their role in the development of cardiovascular disease.

Measurements of vessel remodeling: The mechanisms of vessel remodeling will be assessed by analysis of appropriate parameters before / during or after vascular remodeling.

a) Blood pressure – Tail cuff plethysmography and radiotelemetry are two methods used to measure blood pressure in conscious animals. Tail cuff plethysmography is a non-invasive method that involves placing a cuff around the tail to measure the blood pressure in the tail artery. Radiotelemetry, on the other hand, involves implanting a telemetry probe into the animal to directly measure blood pressure. We will first use tail cuff plethysmography to determine the success of the RUPP (reduced uterine pregnancy pressure) surgery, which is a model for hypertension in pregnancy. Once the RUPP model is established and reproducible, the researchers will then proceed to implant telemetry probes into the animals to monitor blood pressure in real-time. This combination of methods will provide a more comprehensive understanding of the mechanisms of vessel remodeling and the role of vasoactive and anti-oxidative genes.

b) In vivo imaging – Using the expertise and resources available within the institute, we plan to utilize methods that allow for repeatable, non-invasive assessment of vessel remodeling. High quality, in vivo imaging would be achieved via administration of suitable contrast agent. A variety of methodologies will be used to determine the optimal state-of-the-art in vivo imaging technologies for our vascular disease models. A high-resolution ultrasound scan is a diagnostic technique, which uses high-frequency sound waves to create an image of the internal organs and would allow for measurements blood flow in specific areas of placenta.

c) Physical parameters and blood sampling – Samples of blood will be taken to assess circulating angiogenic and anti-angiogenic proteins following viral injection and/or drug concentrations where therapy is given. We may also collect 24h urine from the treatment groups.

Interventions and therapy:

a) Treatments - Therapies may be used that stimulate and/or inhibit or aid in the imaging of vessel remodeling. Treatments may be given before and/or after induction of vessel remodeling by single, repeated, or continuous application.

b) Routes of administration – Treatments may be given by; i) injection (subcutaneous, intravenous, intraperitoneal, intramuscular), ii) inhalation, iii) orally (ad libitum in food/ drinking water or by gavage),

iv) mini-pump implantation, v) subcutaneous pellet implantation, vi) topical application to the skin. vii) Cornea micro-pocket assay.

Adequate measures will be taken to minimize pain and distress, and every effort will be made to provide the best possible conditions for the animals. Pain relief measures will be provided where necessary, and animals will be euthanized in a humane manner at the end of the experiments, if required.GA animals bred under this PPL may also be used under other PPLs with the appropriate authority.

What are the expected impacts and/or adverse effects for the animals during your project?

The experiments planned in this project aim to investigate the mechanisms of preeclampsia and cardiovascular diseases and evaluate potential therapeutic options. To do this, a variety of animal models will be utilized to demonstrate the vascular aspects of these diseases, such as hypertension, elevated pro-oxidant proteins in circulation and onset of renal disease. These models will provide a platform for investigating the underlying mechanisms of regulation of new blood vessel formation and provide a way to evaluate potential therapeutic options.

The animal models will be subjected to a range of procedures, including viral infections, drug treatments, and implantation of devices to deliver substances. Most treatments will be given via intravenous or intraperitoneal injections and are not expected to result in any adverse effects. However, these procedures may result in momentary discomfort or pain, which will be treated with appropriate pain management strategies. In addition, surgical procedures will be performed under general anesthesia, and animals are expected to demonstrate normal behavior within a day of the procedure, although mild pain or discomfort may be present, which will be controlled with painkillers.

When measuring blood pressure in mice, the animals will be restrained, which will result in brief discomfort, but is unlikely to cause long-lasting harm or stress. The length of the experiments will vary, with studies in pregnant mice lasting for the duration of the mouse pregnancy (19 days), and studies investigating the role of angiogenic genes on the cardiovascular system being conducted for between 4 and 12 weeks.

It is not expected that these models will result in serious adverse effects or abnormal behavior. However, if any adverse effects are observed, they will be monitored and treated appropriately to minimize any suffering.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

In the study, most of the procedures performed on the animals will not cause significant harm or stress, and therefore have a mild severity classification. The transient mild discomfort associated with these procedures will likely include brief restraint during blood pressure measurement, as well as momentary discomfort during injections.

For some of the animals, however, repeat procedures may be necessary to assess blood flow, which can move their classification to moderate severity. Additionally, animals undergoing general anesthesia may also experience a moderate severity classification, due to the duration and nature of their recovery period.

It is important to note that any procedures causing moderate or severe distress to the animals will be performed in accordance with ethical guidelines, and steps will be taken to minimize suffering and promote animal welfare. This may include the use of pain control medications and monitoring the animals for any adverse effects following the procedure.

What will happen to animals at the end of this project?

- Killed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In our opinion, animal models are necessary to study the complex mechanisms of vascular remodeling and angiogenesis, and the effects of various treatments on these processes, in a more realistic and biologically relevant manner. The use of animal models is considered essential to the aims and objectives of this project and no alternative methods have been found that would allow us to achieve these goals.

Which non-animal alternatives did you consider for use in this project?

The use of animal models is necessary for the success of our research in this project. Although in vitro methods may provide some supplementary information, they are not able to fully replicate the complex and dynamic in vivo environment. The integrative nature of in situ tissue function and the differences between in vitro and in vivo processes make the use of animal models an essential component of our research. Our research is unique in its ability to study these factors in vivo, and the observational studies in animals will form the foundation for our work. However, we recognize the importance of minimizing animal use and will strive to replace animal studies with non-animal methods whenever possible.

Why were they not suitable?

In this project, the study of whole organisms is essential for the scientific understanding of placental and vascular systems that control resource allocation. The interplay between the mother and the placenta or the development of the vasculature during wound healing can only be fully understood using physiological assays in living mice.

While in vitro systems are useful for investigating molecular pathways, they do not have the level of complexity that is required to study the behavior and interaction of different cell populations in a living animal. This is a crucial aspect of our research as it is not possible to understand the whole-body complexity in cell culture-based experiments.

To reduce the number of animals required and to optimize experimental design, we will run experiments in cells as much as possible. In addition, we will use appropriate human term placental samples to translate our mouse studies into human populations. This is important for the translation of fundamental biomedical research into clinically useful products or protocols.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimize numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The number of animals to be used are usually set with the help of a mathematical model. It allows us to calculate the minimal number of animals we need to use. For a particular experiment, to find differences between groups of animals, whilst making sure that those differences we may find are not due to chance.

In addition, we make every effort to reduce the number of animals used by using optimal experimental design and refining our procedures. We also use appropriate statistical

analysis to ensure that the smallest number of animals are used to provide statistically meaningful results. In line with the 3Rs (Replace, Reduce, Refine) principles, we constantly evaluate and refine our methods to ensure that the use of animals is minimized and that their welfare is maximized. Additionally, we follow the guidelines set by relevant animal welfare organizations to ensure that all animal use follows the highest ethical standards.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

To optimize the number of animals used in the project, we will adopt and implement the 3R principles, make as many observations/measurements as possible on individual animals, and use ex-vivo and in vitro methods where appropriate. We will also use efficient breeding regimes, perform pilot work in cells growing in a dish, and follow local and national guidelines for experiment design and data preparation / publication. Additionally, we will write comprehensive study plans for each experiment to ensure the ethical and scientifically robust conduct of the research while minimizing the use of animals to the greatest extent possible.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimize welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We are committed to adhering to the highest ethical standards in our research and will take all necessary measures to minimize animal usage and ensure the well-being of the animals used in our studies. In addition to our experience and expertise in the use of these models, we will utilize the 3R principles of Replacement, Reduction, and Refinement in all aspects of our work.

To minimize the discomfort, pain, and stress experienced by the animals, we will employ humane endpoints, refine surgical techniques, and use appropriate anesthetic and analgesic protocols. Our goal is to carry out the research in a scientifically robust and ethical manner, while minimizing animal usage to the greatest extent possible.

We will continue to write comprehensive study plans for each experiment and keep detailed records of our methods and observations, to ensure that our research is

scientifically rigorous, transparent, and in compliance with relevant ethical standards. We are committed to working with the NC3R and other relevant organizations to promote the responsible use of animals in research and to develop new, innovative methods that reduce the need for animal usage while maintaining the scientific validity of our results.

Why can't you use animals that are less sentient?

We have not considered the use of less sentient animals, and mice being the least sentient in which our work can be carried out. To achieve our objectives, we need to use a mammalian species that have a placenta and that can be manipulated genetically.

Mice will be the major targets because the use of transgenic and knockout technology. The use of genetically altered animals is critical to this programme of work in that, in nearly all cases, this is the only way to establish the functional role of a protein in supporting angiogenesis. Dose setting studies will be undertaken, at a low dose in no more than two animals initially. Pharmacological dose will be selected based on previous animal studies with the type of compound, or by extrapolation from in vitro work or other appropriate data.

How will you refine the procedures you're using to minimize the welfare costs (harms) for the animals?

To improve the quality of life of the animals, we will continue to:

Reduce contingent harm by group housing where possible to keep singly housed mice to a minimum. This in turn reduces any stress and stereotypical behavior.

Use environmental enrichment EE, within what is available to us at our animal facility. In general, EE is an animal housing technique composed of increased space, physical activity, and social interactions, which in turn increases sensory, cognitive, motor, and social stimulation. Igloos, running wheels, saucer wheels, fun tunnels, and other objects in the housing environment foster this sensory cognitive, social, and motor stimulation by promoting exploration and interaction. EE can be maintained through restraining (e.g., Handling tunnels), thus minimizing stress when for example an injection is needed.

Keep animal transportation to a minimum (majority of our mice are bred in house and kept in one facility).

Use analgesics to lessen pain.

Provide 'behavioral' training to mice undergoing specific procedures (e.g., blood pressure measurements by tail cuff; acclimatization to hypoxic chambers; acclimatization to metabolic cages)

Use scoring sheets to monitor the health of animals undergoing procedures.

Use mash, with improved nutritional composition/palatability, for animals likely to lose weight- e.g., before they start a treatment where weight loss is expected and kept for the duration of the procedure.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Laboratory Animal Science Association (LASA) guiding principles documents of aseptic technique (https://www.ubs.admin.cam.ac.uk/files/lasa_aseptic_surg.pdf)

ARRIVE (Animal Research: Reporting of In Vivo Experiment) guidelines for preparing papers for publication (https://www.nc3rs.org.uk/arrive-guidelines)

PREPARE (Planning Research and Experimental Procedures on Animals: Recommendations for Excellence) guidelines for planning our experiments (15 topics including formulation of the study, dialogue between scientists and the animal facility, and methods) (https://www.ncbi.nlm.nih.gov/pubmed/28771074).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will regularly consult the NC3R's website and engage fully with the regional programme manager of the NC3R's to ensure we are kept informed of the latest developments and opportunities to implement new alternatives/refinements; an example of this is our commitment to the recent drive by the NC3R's to avoid picking the mice up by the base of the tail, even for brief periods, which has been shown to increase levels of anxiety/stress. In addition, we will keep up to date with relevant literature within the field, including attendance at research conferences, to refine our experiments and/or reduce/replace the animals we use. When alternative models or approaches are available which represent an improvement upon current practices, we will perform studies (including pilot studies) to confirm their effectiveness and ensure training for all users and adopt these approaches.

190. Role of natriuretic peptides in cardiovascular homeostasis and disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Natriuretic peptide, Cardiovascular, Homeostasis, Disease, Therapy

Animal types	Life stages
Mice	embryo, neonate, juvenile, adult, pregnant
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

This programme of work will investigate the physiological and pathological actions of a family of substances produced by the body, natriuretic peptides, in the cardiovascular system and how targeting these peptides might be of therapeutic benefit.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Cardiovascular disease (CVD) is the leading cause of death and disability in the UK and accounts for a significant proportion of total healthcare costs. Whilst the introduction of targeted therapies over the last decades (e.g. anti-hypertensives, anti-platelet, lipid-lowering) has reduced this burden, significant mortality remains alongside increasing levels of morbidity. The programme of work described in this project licence will provide information that will enhance our understanding of the mechanisms involved in the maintenance of normal cardiovascular function and give insight into the pathogenic mechanisms responsible for CVD, with the ultimate goal of identifying novel therapeutics to treat these disorders.

What outputs do you think you will see at the end of this project?

1. Generation of unique strains of mice that are genetically modified to delete members of the natriuretic peptide family of hormones and their cell-surface receptor proteins. Investigation of these mouse models will provide insight into the mechanisms underpinning cardiovascular disease (CVD) and reveal new pathways involved in the inherent protective functions of natriuretic peptides in the cardiovascular system, potentially involving new systems (e.g. immune cells, fat cells, neurones).

2. Development of new treatments for CVD. The major goal of our research is to identify novel therapies for CVD, based on a greater understanding of the pathogenic mechanisms underpinning these disorders, with specific focus on the actions of natriuretic peptides. Thus, we will undertake investigations exploring the functional pharmacology of interventions targeting natriuretic peptide signalling in realistic models of human CVD. To this end, we have an active academic drug development programme in progress to identify novel small molecules modulating natriuretic peptide signalling.

Who or what will benefit from these outputs, and how?

Academic research: The findings from our studies will guide future research to better target CVD.

Industrial research: A better understanding of the biology of natriuretic peptides will aid the development of new medicines in the pharmaceutical industry

Clinical medicine: Results from this work should provide a better understanding of the pathogenic mechanisms underpinning CVD thereby helping with the diagnosis and prognosis of such disorders, including the implementation of existing and new medicines targeting natriuretic peptides.

How will you look to maximise the outputs of this work?

1. Towards the end of this programme of work we anticipate to have several publications in press to share our findings with both the scientific and medical communities. We also frequently attend professional meetings to keep up to date and discuss our findings.

2. We will engage actively with members of the lay public and patient population through events such as 'Pint of Science' and 'Let's talk hearts'.

3. We have ongoing collaborations with both national and international teams focused on CVD research. Through these collaborations we share data including problems, unsuccessful approaches, and the development of better animal models of disease.

Species and numbers of animals expected to be used

- Mice: 9000
- Rats: 750

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

This proposal adopts a multidisciplinary approach to elucidate the role(s) natriuretic peptides play in the pathogenesis of CVD that will require validation in isolated cells, tissues, and in experimental models of disease. There are no existing well-validated alternatives to using laboratory animals for such studies. However, a sizeable portion of the work will be carried out using cell-based assays (including human samples) thereby limiting the number of animals used. The project involves the use of mice and rats since the applicant has considerable experience and expertise in pharmacological experimentation in rodent models. Moreover, rodents have been used extensively for modelling many aspects of CVD since they closely mimic the human pathobiology and are well-characterised. Finally, the use of mice allows exploitation of knockout technology which is key in the setting of natriuretic peptide biology since, whilst pharmacological tools exist, they are limited in terms of potency and selectivity. We have developed in-house several unique transgenic strains for this purpose, which will be widely utilised in this project. Whilst the vast majority of the work will involve the use of mice, the project necessitates the use of rats for a number of important reasons, including differential actions of natriuretic peptides in the microvasculature of rats compared to mice, more apparent phenotypes in rats in certain models (e.g. right ventricular hypertrophy in pulmonary hypertension) enabling more focused study, and the need for rat functional pharmacological data for novel therapeutics that will align with downstream pharmacokinetic/toxicological workups.

Typically, what will be done to an animal used in your project?

Animals will undergo ONE of the models of cardiovascular disease (e.g. heart failure, pulmonary hypertension, cardiometabolic disease) permitted. In addition, some animals will receive existing or novel pharmacological interventions selectively targeting natriuretic

peptide signalling which are not anticipated to have a significant adverse effect profile. A small cohort of animals will also undergo implantation of catheters or radiotelemetric devices to permit longitudinal study of cardiovascular parameters in as least invasive manner as possible. We will use non-invasive methods of assessment (e.g. imaging) to determine the structure and function of the heart and blood vessels. Model duration and experimental treatment are relatively short, typically 12 weeks maximum. Animals will be appropriately anaesthetised for any surgical interventions with close monitoring and appropriate analgesic use post-surgery. Aseptic techniques will be used routinely. During the 5-year period for the project we anticipate the use of approximately 9000 mice spread over nine protocols.

What are the expected impacts and/or adverse effects for the animals during your project?

The experimental models utilised in this project are classified as mild or moderate. The animals tolerate these procedures well and, although this is a marked cardiac and/or vascular impairment, there are no overt signs of stress/adverse effects. These experimental models are not associated with mortality since the dietary interventions, genetic modifications and surgery are well refined and extensively used in my group and the time course relatively short term. Regardless, any animal showing signs of adverse effects will be discussed immediately with the named veterinary surgeon (NVS) and/or named animal care & welfare office (NACWO) and monitored closely and/or administered appropriate analgesia, or killed by a Schedule 1 method, whichever is deemed necessary.

Expected severity categories and the proportion of animals in each category, per species.

- What are the expected severities and the proportion of animals in each category (per animal type)?

In the breeding protocol we do not expect any harmful effects from the genetically modified strains we will investigate and from experience these protocols are therefore of mild severity with the majority of animals experiencing sub-threshold severity. All other protocols have either a mild or moderate severity. We do not expect any severe effects in our protocols.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Numerous cell-based techniques are now available to aid the development of new classes of drug for the treatment of CVD. However, because of the dynamic cellular interactions that typify the initiation and maintenance of cardiovascular pathology, to facilitate the identification and subsequent development of such agents it is also necessary to use animal models. Whilst there are few animal models that faithfully reproduce all the pathology of the analogous human condition, it is possible to produce certain fundamental processes that may either reveal new targets or act as screens for drug testing. Thus, provided one is aware of the limitations of animal models they can play a valuable role in the development of novel and more efficacious drugs.

Which non-animal alternatives did you consider for use in this project?

In order to minimise animal usage, we are utilising highly sophisticated cell- and tissue-based models (e.g. cell-based assays, organ-on-a-chip), and cells/tissues from patients with CVD. We are also integrating our laboratory research with the extensive bioinformatics platforms available at the Institution that enables us to refine interventions/targeting before administration to whole animals. Only new interventions that demonstrate repeated efficacy in these early-stage studies will be further examined in live animals. We have already minimised the number of animals that will be needed by developing these non-animal models and will continue to improve and expand these during the course of this project.

Why were they not suitable?

Although these cell- and tissue- based techniques are the most sophisticated models of cardiac and vascular function that are available, they fail to model critical aspects of the live animal situation. These include pharmacokinetics, pharmacodynamics and interactions with other organ systems. These aspects can only be assessed in representative models of CVD in whole animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Sample sizes have been calculated according to our previously published work and pilot data on the effects of natriuretic peptides on vascular & cardiac function. Total numbers are based on the planned studies using appropriate power calculations and the usage, in matching models, during the 5 year tenure of a previous licence.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

By undertaking studies with adequate statistical power, definitive positive or negative data can be generated from a single experiment, thus avoiding under-powered experiments that may be futile and wasteful. The minimum numbers of breeding pairs will be mated to provide offspring to enable experimentation. Working on the basis that a breeding pair produces a litter once a month of approximately 8 animals and that 50% of these are WT or KO, we would need approximately 4-6 breeding pairs to provide a minimum of 1 animal per day for experimentation.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

The studies will only include interventions that have been identified using our sophisticated and comprehensive. cell- and tissue- based models of cardiovascular physiology and pathology. We have incorporated whole animal imaging in many of protocols to enable smaller cohorts of animals to be observed longitudinally throughout an experiment rather than relying on killing of separate groups of animals at each time point. By first exploring conditions in small pilot experiments the final conditions can be optimised and also reduce the number of animals subjected to unexpected side-effects. When possible, the same groups of control animals (untreated or sham-treated) will be used in several studies to minimise the number of animals used. Tissues/plasma samples will be harvested from each study for laboratory investigation to collect all available information from each animal.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

All the models described in this licence are mild to moderate; there are no models that fall into the severe category. Each is well-validated and widely-used for investigation of the mechanisms underpinning CVD, and gleans robust and informative information with minimal animal suffering; analgesics and anaesthetics are used whenever necessary, and close monitoring of all animals undergoing licensed procedures is undertaken.

Specifically, sepsis (a systemic inflammatory/immune response) can be induced by the administration of lipopolysaccharide or by caecal ligation and puncture. Two other CVDs in which natriuretic peptides have been implicated are pulmonary hypertension and heart

failure, and we can investigate these diseases using established techniques that involve subjecting animals to chronic hypoxia that results in the progressive development of pulmonary hypertension which is associated often with an outcome of heart failure. But we can look more specifically at heart failure using the technique of aortic banding (to create a back pressure on the left ventricle resulting in enlargement), by administration of vasoconstrictor agents or by closure of the left anterior descending coronary artery to induce a heart attack (leading to heart failure). Predominantly dietary interventions will be used to study cardiometabolic and liver disease.

Why can’t you use animals that are less sentient?

These models are the most representative of the human disorders available and reflect the most up-to- date information about human CVD. More immature life forms are not suitable due to the significant differences between such species (e.g. worms, flies, and fish) and humans and, and the lack of relevant circulatory (and immune) systems.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

In the course of the current project we have significantly refined our procedures with the help of the NVS, the facility managers and the animal technicians. Surgical procedures and postoperative care including pain management are always discussed with the NVS if the study has not previously been performed. Another refinement is the continued and increased use of imaging in our studies that enables us to follow each animal over longer periods of time rather than harvesting tissues from numerous animals at several timepoints, thereby maximising the information obtained from each animal.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Curtis et al. 2018. Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Br J Pharmacol. 175:987-993. doi: 10.1111/bph.14153.

Smith et al. 2018. Classification and reporting of severity experienced by animals used in scientific procedures: FELASA/ECLAM/ESLAV Working Group report. Lab Anim. 52:5-57. doi: 10.1177/0023677217744587.

Charan & Kantharia. 2013. How to calculate sample size in animal studies? J Pharmacol Pharmacother. 4:303-6. doi: 10.4103/0976-500X.119726.

All recent advances and practices on NC3Rs website (https://www.nc3rs.org.uk/). The Home Office regulations (online material).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

PIL holders working under this licence will regularly attend NC3R or similar meetings to keep abreast of new developments, and to maintain awareness of current best practices. The applicant is on the NC3Rs mailing list and are regularly informed of seminars and workshops that train users in new techniques, and inform on regulations. We receive regular updates on new applications and regulations at an Institute-wide users committee and from the NVS.

191. Vaccine candidates for group a streptococcus infections

Project duration

2 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Group A Streptococcus (Group A Strep, GAS), Vaccine, Immune response, carbohydrate

Animal types	Life stages
Mice	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The overall aim is to identify vaccine candidates that can induce a strong and specific immune response, ultimately for the treatment of Group A Streptococcus infections.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Our research addresses the prevention of human infections caused by the human-exclusive pathogen Group A Streptococcus (GAS). GAS causes a range of mild and severe diseases such as scarlet fever and streptococcal toxic shock, with more than

500,000 deaths annually worldwide. The effectiveness of current treatments for some infections is waning because of antibiotic resistance and the emergence of hypervirulent strains (those that can adapt to cause organ-invasive serious infections). In 2013 there were more than 1400 cases reported of invasive GAS infection in the UK alone, with mortality rate of > 30% within 30 days. To date, no vaccine has been successfully developed against GAS. This is due to the high number of GAS variants (serotypes), meaning that there are too many targets to generate a specific vaccine. However, a molecule that is common to most of these variants has now been identified and we wish to explore the possibility that it will be a good target for vaccination.

What outputs do you think you will see at the end of this project?

The main outputs from this study are expected to be one or two vaccine candidates validated to trigger a strong and specific immune response to GAS species, to take them further into additional preclinical trials. Importantly, this is also the first approach that has the potential to target other closely related bacteria. The findings from this study will be published after peer review in an international (open access) journal.

Who or what will benefit from these outputs, and how?

In the short term, these outputs will benefit justification to conduct subsequent research studies (preclinical trials). The studies will obtain knowledge of immunogen/vaccine candidate effectiveness to trigger specific immune response. In the long term, these vaccine candidates will have a potential benefit to the patients, preventing infections in humans. Should the proposed work lead to the development of one more more efficacious vaccine candidates (e.g. prevention and eradication of bacterial disease in the animal model (late preclinical studies), they will be tested in humans clinical trials.

How will you look to maximise the outputs of this work?

We have established collaborations with experts in this area of research, who are also co-applicants and collaborators on the 2-year funded grant. The main outputs of the studies will be peer reviewed publications in scientific journals and attendance at national and international conferences.

Species and numbers of animals expected to be used

- Mice: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Because of the very significant similarities in the immune systems of mice and humans, the mouse is a good model in which to test vaccine efficacy. There are also excellent reagents and procedures with which to test the immune response in the mouse. We will use adult mice from around 10 weeks old, when the immune system is fully mature.

Typically, what will be done to an animal used in your project?

Typically mice will be injected with a vaccine candidate, together with a suitable adjuvant (a substance intended to ensure a robust immune response) and boosted at intervals thereafter, much as human vaccinations are performed. Blood samples will be taken at intervals to monitor antibody levels and other indicators of an immune response. Mice will be killed humanely at the end of the experiment.

What are the expected impacts and/or adverse effects for the animals during your project?

We have previously performed pilot studies and have optimised the composition of the adjuvant to avoid adverse effects. We do not anticipate any adverse effects from the vaccine candidates.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

We expect the severity experienced by all the animals to be "mild".

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

In order to develop and test vaccine candidates, we need to challenge an intact immune system, which cannot yet be modeled satisfactorily without using a living animal.

Which non-animal alternatives did you consider for use in this project?

We considered the following models:

1) Reconstituting the immune system in sufficient detail and complexity in a cell culture dish and testing vaccine candidates in this model;

2) Moving straight to studies using human volunteers would be an excellent system in which to test our vaccine candidates;serve as an excellent model to study the role of the glycoconjugate vaccines against Group A Streptooccus (GAS), as they naturally colonise and infect only humans.

Why were they not suitable?

The immune system cannot yet be reconstituted in a cell culture dish in sufficient complexity to produce the types of responses seen in intact animals and humans (for example, we cannot "input" a vaccine in a cell culture system and measure antibody production as an "output"). Direct human trials will not be not feasible until there is sufficient evidence of safety and the ability of the vaccine candidates to induce the desired response in animal models.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We will perform a number of small pilot studies to validate, optimise and screen vaccine candidates and compare them, where applicable, to recently reported candidates.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Our preliminary pilot studies have allowed us to estimate the likely variation in immune responses and to choose a default group size for future experiments. Further small-scale studies with new candidate vaccines will ensure that we can optimise the dosing regimes and the number of animals in each experimental group.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will share tissues with other groups highly experienced in dissecting the immune response to aid us in our investigations.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the

mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

We will use a standard mouse model of immunisation to induce an immune response. The use of adjuvant and subsequent boosters may be required to enhance the immune response to the vaccine candidate, but we do not expect these to cause significant adverse welfare effects.

Why can’t you use animals that are less sentient?

We require adult mice in which the immune system is fully mature and to keep these animals for a few months in order to measure the development of the immune response to a vaccine candidate.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will use clinical scoring systems in order to monitor mice during experimental phases. Mice will be handled in advance of starting experiments, in order to acclimatise them. Procedures will be carried out by experienced and competent staff.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We follow the NC3Rs for best handling and procedure practise, and will adhere to LASA guidelines on dosing.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I receive the NC3Rs email updates on recent advances, as well as staying informed via our local NIO and animal users group forum.

192. Novel mechanisms of co2 signalling in the physiological systems

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

breathing, neuropathy, neural circuits, metabolism

Animal types	Life stages
Mice	adult, juvenile

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

We wish to understand how CO2 can act as a signalling molecule to control physiological processes.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

CO2 is an unavoidable by product of cellular metabolism. Historically, CO2 has been regarded as a waste product rather than a molecule that carries information. Even for physiological processes that are well known to be “CO2 sensitive”, such as breathing or cerebral blood flow, the actions of CO2 are often considered to occur only via consequent changes to pH. This is because CO2, through its ready reaction with water and production of bicarbonate and hydrogen ions, is a prime determinant of physiological pH.

Our work, over the past 10 years, has changed this by identifying a small group of membrane channels called connexins that directly interact with CO2, which controls their opening and closing. The CO2 sensitive connexins are expressed in many organs of the body including brain, liver, kidney and skin. Mutations of these connexins cause a range of pathological conditions that include hearing loss, fatal syndromes such as Keratitis Ichthyosis Deafness Syndrome (KIDS), and neurodegenerative diseases that include X-linked Charcot Marie Tooth disease. Some of these pathological mutations affect the CO2 sensitivity of these connexins, suggesting that this property could be important physiologically and its loss could contribute to disease. We have already shown that one of the CO2-sensitive connexins, Cx26 plays a key role in the sensory control of breathing. This programme of work seeks to uncover further roles of CO2 signalling mediated by the connexins. Given the pivotal role of connexins in human health and disease, our work is likely to provide better knowledge of the mechanisms of disease and hence enable earlier diagnosis and better treatments

What outputs do you think you will see at the end of this project?

1. Creation of fundamental new knowledge concerning the control of physiological processes such as breathing, arousal, maintenance of myelin via CO2 signalling including identification of the molecules that are critical to this process and the neural cells and circuits that are involved.

2. Molecular and genetic tools that will allow analysis of CO2 dependent signalling. These will include methods to directly measure the production of CO2 in tissue with subcellular resolution, and to alter the CO2 sensitivity of endogenously expressed connexins to test the role of CO2 dependent signalling in physiological processes.

Who or what will benefit from these outputs, and how?

<u>Primary benefits</u>

1. Dissemination of novel information by publishing papers in international journals and presenting our findings at scientific meetings and conferences. This will benefit the research community as the outcomes of this project will provide significant advances in knowledge in the following fundamental areas of neuroscience: understanding the mechanisms of how CO2 triggers arousal and wakefulness; the neural circuits and signalling controlling breathing; and the signals that are required for the development and maintenance of healthy myelinated nerves.

2. The research community will benefit from the molecular and genetic tools that we generate and plan to make available for use by others. These will be validated by our own work and will enable others to use them in their own discovery-led research. For example the genetically encoded sensors will be critical for others to examine the production of CO2 other physiological contexts and even other organ systems.

<u>Secondary benefits</u>

1) **Pharmaceutical industry**

The results generated from this project will be of significant interest to the pharmaceutical industry in respect of identifying potential new targets for drug development (connexins) and new ways to target them. Our work will provide understanding for the scientific basis of pharmaceutical intervention in respect of the pathophysiological outcomes of connexin mutations which will manifest as central apneas (with resulting comorbidities such as enhanced risk of diabetes and cardiovascular disease), defects of arousal from sleep, and diseases of myelin.

2) Government, society and economy

Connexin mutations are relatively common. For example mutations of Cx26 cause deafness in ~1:3000 people. Mutations in Cx32 cause X-linked Charcot Marie Tooth disease (CMTX, a peripheral sensory and motor neuropathy, sometimes accompanied by cognitive deficits). In the UK about 25,000 people are estimated to have CMT making it the commonest inheritable neurological condition and about 10% of these will have the X-linked form caused by Cx32 mutations.

Our data shows that some of the pathological mutations affect the ability of these connexins to detect CO2. In the case of Cx26, this implies that the CO2-dependent processes such as control of breathing, and arousal will be altered in these people and they may be at greater risk of developing sleep apnoea and consequent comorbidities. Our work could help to predict these outcomes and in turn could lead to timely preventative treatment that would improve their quality of life and have a large impact on the NHS. For Cx32, our work may permit better stratification of risk in CMTX patients, and possibly tailored treatments depending on the nature of the mutations and their effect on the molecule.

How will you look to maximise the outputs of this work?

Dissemination of results and knowledge to the scientific community via scientific papers and meetings. Putting novel resources onto searchable databases and repositories so that others can access and use them.

Collaboration with other groups to assist them in applying our methods, resources and tools in their own research. Dissemination of knowledge to the wider community through public engagement activities, engagement with relevant charities (e.g. Deafness, CMTX).

Press releases on publication of key findings that will have major impact.

Species and numbers of animals expected to be used

- Mice: 1000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We use mice throughout the project as they are an excellent genetic model. This gives the advantage of a rich suite of genetic strategies to target specific cell populations in the brain and nervous system either to specifically record their activity, manipulate their activity, or alter their gene expression. These strategies are essential to allow rigorous analysis of the underlying mechanisms. Genetic manipulation of mice to introduce key mutations in connexin genes or to delete these genes recapitulates the pathologies that occur in the human diseases associated with mutations in these genes.

Typically, what will be done to an animal used in your project?

A small hole will be made in the skull to enable precise injection of virus particles to give expression of a gene that will: delete expression of a key gene or genes in a specific cell population; introduce altered function of endogenous genes; activate or inhibit populations of cells to test their function; or report the activity of the cells that it is expressed in by its intensity of fluorescence.

Where possible the mouse will be killed under terminal anaesthesia to provide tissue to allow us to study signalling mechanisms and function in vitro. Where this is not possible, because for example we need to study behaviour such as breathing or arousal from sleep, we shall keep the mouse for up to two months post surgery. During this time we will use a variety of non-invasive tests to measure arousal or breathing to examine the effect of our manipulations.

In mice where we need to directly measure the activity of neurons or other cells during behaviour, 2 weeks after recovery from injection of viral particles, another hole will be made into the skull and a special lens implanted into the brain so that the fluorescence from active cells can be measured. At this time electrodes may also be implanted to allow the recording of brain waves. The mouse will be allowed to recover and a further week later a special baseplate will be glued to the head in a final minor surgical procedure. This baseplate allows mounting of a minimicroscope that will allow recording of neural activity as the mouse behaves normally. We shall keep the mouse for up to 2 months following the final procedure to allow behavioural and physiological testing.

What are the expected impacts and/or adverse effects for the animals during your project?

The surgical procedures cause short lasting pain that will be mitigated using analgesia, but following recovery there are minimal adverse signs. Our behavioural testing is non-

invasive and does not cause adverse effects. We do not expect the viral genetic manipulations to cause harmful phenotypes.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The expected severities are moderate in all animals.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

This project seeks to understand: the contribution of CO2-sensitive connexins to functioning of the nervous system –specifically CO2-evoked reflexes involved in the control of breathing and arousal; axon-myelin communication in the context of the causes of peripheral neuropathies. As these are phenomena that arise in the whole organism they can only be studied in whole animals and tissues taken from animals. Rodents are generally accepted as being good models for these aspects of human physiology. It would be unethical to use human subjects in these experiments.

Although we will still need to perform the viral transduction procedures to label, manipulate or monitor key populations of cells, we shall also use ex vivo preparations to assess the effects of these manipulations as much as possible. Additionally, we have replaced use of animals in our molecular/biochemical investigations. For example our use of the HeLa cell expression system will allow us to the test the effects of connexin mutations prior to development of viruses for in vivo experiments. Together this combination of ex vivo and in vitro work will act as a replacement, minimizing rodent numbers by only using effective mutations and genetic tools and remove risk from subsequent work on whole animals.

Which non-animal alternatives did you consider for use in this project?

Cultured cell lines, organoids, computer modelling.

Why were they not suitable?

Cultured cell lines. While these can be useful for examining properties of mutant connexins or evaluating genetic tools prior to use in vivo (and we use them for this), these cells

cannot reproduce the complete physiological functions such as breathing, arousal, nerve conduction that we wish to study.

Organoids. These have not reached the level where they can reproduce the mechanisms and interacting systems that we need to study to advance the field.

Computer modelling. All good models require data to allow them to be realistically specified and hence useful as predicted tools. We simply lack sufficient data to enable this approach as a realistic way forward.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have considered the range of experiments and manipulations that we need to perform to advance knowledge. Using information from our prior work or the work of others we can calculate the numbers we need to give definitive results that we can be confident will be reproducible by others.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We used the best estimate of effect size based on pilot data or published literature to inform our power calculations. As the behavioural tests we use are non-invasive, we can use a "repeated measures" design to increase the confidence in our data without causing suffering, and hence use this design to reduce overall animal usage.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We shall ensure all personnel are highly trained and skilled so that very few experiments will fail for technical reasons.

Pilot studies will be performed to get good estimates of effect size, feasibility, and ensuring that all technical problems have been anticipated and solved. Should our program demonstrate that our estimates of effect size are wrong (either too big or too small) we shall reperform the power calculations to ensure we arrive at a rigorous outcome. If it appears that the effect size is much smaller than anticipated for a particular experiment, we shall consider abandoning that part of the program. We shall routinely harvest tissue to

enable biochemical, genetic, cellular and morphological analyses and share this tissue with our collaborators.

We shall use efficient breeding strategies to minimize the number of animals that are bred. In some case we may have to maintain lines as heterozygotes meaning that animals (which do not carry the gene of interest) cannot be used. Where at all possible such animals will be used for control comparisons giving the benefit of identical genetic background, however this means that we will unavoidably breed more animals than we will use in procedures.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Rodents are a well-established model for studying the breathing and arousal. They are also used to examine mechanisms of myelination and the effects of connexin mutations more generally. This gives a robust literature and wealth of potential data obviating the need to repeat past findings. Rodents in general are seen as a simplified and experimentally tractable model for far more complex mammals such as humans.

Wherever possible, physiological testing of phenotypes will be achieved by using non-invasive methods such as: whole body plethysmography for detection of breathing movements. More invasive procedures will be used only when there is good reason (from the non-invasive experiments) to expect mechanistic insight.

We shall use ex vivo tissue as much as possible to study cellular mechanisms relating to the neural circuits relaying chemosensory signals, and axon-myelin communication. This will enable future in vivo experiments to be appropriately designed to minimize animal usage.

Why can’t you use animals that are less sentient?

We wish to examine these phenomena in a mammalian system to have maximum applicability to the human condition. By choosing mice, we have chosen a model that has the lowest sentience.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We have considerable experience with the in vivo imaging methods and have considerably refined them already over the past 2-3 years. We have implemented standardised post-operative monitoring methods to ensure that outcomes and adverse effects from our surgical procedures are carefully assessed. We use this information to optimise post-operative care and pain management.

We shall use rigorous aseptic techniques to protect against infection of the animals.

We train the animals with a dummy camera to acclimate them to the imaging procedure and to the imaging cage to minimize stress.

As imprecise targeting of viral injections can increase animal usage, we shall be very careful in this aspect of our procedure. We shall ensure we use calibrated stereotaxic frames, check the alignments thoroughly and regularly use the injection of fluorescent beads (in a non-recovery terminal procedure) to ensure that correct targeting is achieved.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We shall take careful note of advice from collaborators and researchers highly experienced in these methodologies to ensure that we refine our experiments as much as possible. We shall follow the Laboratory Animal Science Association guidelines on performing aseptic surgery to ensure best practice for the recovery surgery involved in all surgical procedures.

To ensure effective and rigorous reporting of our results, we shall write papers according to the ARRIVE guidelines which are recognised as providing excellent transparent standards for reporting of research using animals and were developed by the NC3Rs.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We receive regular communications from NC3Rs and Laboratory Animal Science Association (LASA) via a newsletter, colleagues who advise on best practice, and the NACWO, NTCO, NIO and AWERB at our establishment. We consult the NC3Rs and

193. Nutrition of growing and mature ruminant livestock

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Improvement of the welfare of animals or of the production conditions for animals reared for agricultural purposes
    - Protection of the natural environment in the interests of the health or welfare of man or animals

Key words

Ruminants, Production, Feeds, Efficiency, Methane

Animal types	Life stages
Cattle	adult, pregnant, juvenile
Sheep	adult, juvenile, pregnant

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

There is a need to maintain the home-grown production of foods produced by livestock (i.e., milk and meat) in the UK, to support the agricultural industry (including for non-food products, e.g., novel packaging) and reduce the reliance on imported foods. The aim of this programme is to increase our understanding of the mechanisms that contribute to the inefficient use of feeds by cattle and sheep, and therefore enable us to reduce the output of pollutants such as nitrates, ammonia, and methane while continuing to produce nutritious food for people.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

This project will allow an improved understanding of the interactions between feed and rumen microbes (colonisation, degradation, fermentation, and microbial protein synthesis) that will improve the efficiency of diet utilisation. This will improve the output of ruminant livestock products and help reduce the environmental impact of ruminant agriculture. For example, an improvement in the use of feed protein for meat and milk production will reduce the amount that is excreted, thereby reducing nitrous oxide (a greenhouse gas) and ammonia release into the atmosphere. Improved understanding of the roles of the rumen microbiota has the potential for development of new products for industrial application based on the specialist activities needed for fibre degradation.

What outputs do you think you will see at the end of this project?

The outputs of this project will likely be new information that will be disseminated to farmers, to other scientists, and to policy-makers in government. The information will be published as reports, information sheets, articles in popular magazines and specialist periodicals, and in the peer-reviewed literature.

Who or what will benefit from these outputs, and how?

Beneficiaries of these outputs are envisaged to include, i) the general public and the farming industry, through an improvement in the production efficiencies of meat and milk and associated reductions in pollutant emissions, and ii) government departments (e.g., Defra) and devolved administrations (e.g., Welsh government) with improved knowledge of the consequences of the development of novel feeds and feeding strategies. Ruminant livestock are vital to the livelihoods and nutrition of the global population, by consuming and converting feeds that humans cannot eat into highly nutritious foods that we can eat. However, we know that ruminants contribute to climate change through the emissions of greenhouse gases (methane and nitrous oxide), and we therefore need to continue to develop ways of improving production systems that minimise pollutant outputs and maximise useful products.

How will you look to maximise the outputs of this work?

We always aim to publish the results of work that we carry out in the peer-reviewed scientific literature, regardless of whether the work was deemed successful or otherwise. Some work is funded in collaboration with industrial partners, and even in those cases we aim to publish results, although sometimes there is an agreed delay to this to allow the companies to exploit an intellectual property that is generated. We aim to maximise the availability of publications by publishing open access articles.

This means that the information is available to anyone, not just those individuals or organisations that subscribe to the journals that we publish in.

Species and numbers of animals expected to be used

- Cattle: 400
- Sheep: 400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To understand the benefits, or otherwise, of feeding different diets to animals, and to measure the effects on the production of meat or milk, it is essential to use growing or lactating livestock. The way young animals are cared for in early life can have significant consequences on how they grow or how well they produce milk later in life. For this reason, it is important to be able to work with animals, from just after birth, until they reach maturity and are productive in later life.

Typically, what will be done to an animal used in your project?

A small number of animals, approximately 20, will be fistulated at the rumen (cattle and sheep). Fistulation is a surgical procedure that creates is a hole (fistula) in the side of the animal (similar to a colostomy procedure in humans) to allow direct access to the rumen and its contents. The fistula is maintained by a rubber cannula that seals the hole, but which can be opened when samples of rumen contents are required. Following surgery, these, and other animals used by the project, will be subjected to regulated procedures such as restraining them in stalls to measure feed intake, taking blood samples, and collecting outputs of faeces and urine using harnesses and chutes. Some animals will be used in short-term research that may last for between 2 to 4 months, whereas other animals will be monitored for several years as part of their normal growth and development. These procedures carry a mild level of severity. At the end of the procedures, those animals that have not been surgically modified will be re-homed in the establishment's herd or flock, sold to another farm, or will be sent to slaughter as part of the normal supply chain for human consumption. Those animals that have been surgically modified are a valuable resource, and if their general health and well-being is still good they will be transferred to another project licence. Fistulated animals often live for longer than a cow or sheep on a commercial farm and will be euthanised when their general health and well-being starts to deteriorate, for example with the onset of arthritis.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals may be restrained in metabolic crates or by using neck yokes, which prevent full freedom of movement necessary to allow the collection of data and samples for experimental purposes. This restraint may last from a few minutes during the collection of rumen fluid from fistulated cattle to several weeks during a feeding experiment. Animals are likely to experience pain for a few days following fistulation surgery, although this will

be managed using suitable pain relief medication. Animals that are blood sampled or have substances administered using a needle and syringe (e.g., anaesthetics) may experience mild transient pain when the needle is inserted. The collection of urine from cattle requires the use of equipment that is temporarily glued over the vulva of the animal for a few days - this sometimes causes skin irritation that is treated with a soothing ointment and there is no lasting harm caused by this procedure. Collection of rumen contents using a stomach tube through the mouth may cause temporary discomfort lasting a few moments as the tube is inserted, although this is minimised by temporary restraint of the animal, and the fitting of a halter that enables the animal's head to be appropriately positioned to allow easy swallowing of the tube or probe. The use of equipment to collected breath samples from the animals requires the use of head collars and/or neck collars to attach sampling tubes and collection cylinders; this may initially cause mild distress in some animals for a few minutes but the risk of this is minimised by training them to become used to the equipment before it is used for experimental collections.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The surgery for fistulation, for both sheep and cattle, is a moderate level of severity. Approximately 5% or less of the animals studied will be fistulated - all other animals will only experience procedures that are expected to be mild or sub-threshold.

What will happen to animals at the end of this project?

- Killed
- Kept alive Rehomed
- Used in other projects

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

For some of the work, measurement of growth or milk production of animals fed well-defined diets is required. In addition, whole-body utilisation and partitioning of nutrients between productive and non- productive (pollutant) outputs may be measured. These data cannot be collected using in vitro techniques, and therefore animals must be used for those parts of the work. The rumen microbiota cannot be maintained outside the animal. Therefore, access to fresh, well-characterised rumen fluid samples are essential for the primary research objectives of this project.

Which non-animal alternatives did you consider for use in this project?

We already use some laboratory methods for initial screening of feeds for, e.g., the potential to produce methane when fed to ruminant animals. These methods are very helpful in being able to rapidly and cheaply assess large numbers of feed samples that are available only in small quantities and allow us to prioritise those to produce in larger amounts for further study.

Why were they not suitable?

Although in vitro methods are very useful, they still require the use of fistulated animals to act as donors of well-characterised and consistent rumen fluid. Rumen fluid sourced from, e.g., abattoir, is not representative of that obtained from a live animal because livestock sent for slaughter have feed withheld for a day before. Furthermore, investigations requiring the measurement of growth or milk yields, and samples of meat or milk, need to use live animals to do this.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers have been estimated based on experience of previous experimental work in this area.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The minimum number of animals required to carry out an experiment, using an appropriate experimental design, that produces statistically valid results will be used. The type of experimental design chosen will depend on the animal type and measurements being collected. For lactating dairy cows being offered different dietary treatments, for example, changeover design (e.g., Latin square) experiments are particularly efficient because each animal receives each treatment, and therefore each animal effectively acts as its own control. However, changeover design experiments are not appropriate for some studies (e.g., growth studies), and therefore more animals are typically required per treatment in order to control variation. In this type of experiment, a set of measurement steps may be carried out on animals of different ages (e.g., 4, 6, 9 and 12 months of age) to assess the interaction between an imposed treatment and age.

For all experiments, it is important to consider what data and statistical analysis methods will be used

before the start of the data collection. This ensures that appropriate measurements are made and can be analysed in a statistically valid manner. Consultation with a statistician during the planning of an experiment is important, to make sure that animal numbers are appropriate, and that the latest statistical methods can be employed during the data analysis part of the experimental process.

Statistical blocking of animals during the experimental stage is important to reduce variation caused by known factors, such as breed, age, gender, stage of lactation and parity. Careful choice of animals, and grouping to reduce known variation, is important to help reduce the numbers of animals needed for an experiment.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

Work to be carried out on this project licence will generally benefit from prior knowledge of variation in expected data that will be collected. This helps define the numbers of animals needed for an experiment. This knowledge can be gained from previous work carried out by the applicant, or from studies published in the literature. However, if a particularly novel treatment will be applied, where the effects are completely unknown, a pilot study will be carried out to with a small number of animals to generate data that will be used to inform the design of a larger study using more animals.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

For much of the work the effects of treatments on feed intake, live weight change, milk production, milk composition, and/or nitrogen and methane excretion in/from cattle and sheep is required. Therefore, sheep and dairy cows are the animals most suitable for use in this work.

Assessment of animals prior to fistulation in Protocol 1 is vital to the long-term success of the use of these animals. Animals are initially selected from a larger group on the basis of age, parity, fitness (e.g., no history of mastitis leading to loss of udder quarters), and

knowledge of temperament (e.g. not known to kick while being milked), and candidates for fistulation are housed temporarily in the metabolism unit stalls to ensure they are able to use them comfortably. For example, some animals are not able to easily stand up or lie down in the stalls, and these are rejected before surgery.

Sampling of digesta contents that leave the rumen (and enter the omasum) may be carried out using the omasal sampling technique. This entails inserting a sampling line through the rumen fistula and through the reticulo-omasa orifice. It allows the collection of samples of digesta that have undergone fermentation in the rumen but have not been digested within the omasum/abomasum. This is important for studying, e.g., rumen passage rate (coupled with the infusion of digesta markers into the rumen at known rates), and can be done using animals that have not been fistulated at the duodenum. However, it can only be done in mature cattle because the omasal sampling line must be inserted manually by a technician placing an arm through the rumen fistula. However, the use of this method means that duodenal fistulation is not required.

Why can’t you use animals that are less sentient?

This work needs to be carried out on growing and mature productive animals, to assess the effects of treatments on growth and lactation

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

For those procedures requiring surgery (i.e., gut fisulation), a sedative will be administered to reduce the stress of anaesthesia on the animal. Forrumen fistulation of mature dairy cows, sedated animals will be supported using a belly-strap and a radial block will be induced using administration of local anaesthetic. Rumen fistulation of young cattle and of sheep will be carried out under general anaesthesia induced and maintained by inhalation following tracheal intubation. The sedation and anaesthetic regime will be devised and refined under the advice and supervision of a veterinary surgeon. To ensure correct operation of the anaesthetic machine, it is serviced and calibrated by a qualified specialist anaesthetic equipment technician prior to a series of surgeries.

Fistulation of the rumen in adult cows is carried out under sedation and local block anaesthesia. Recovery from this is much quicker than from general anaesthesia, so normal behaviour tends to resume more quickly (e.g., feed intake) and a lactating cow can be returned to a stall and be milked there. Surgically modified cows are not run with the main herd cows and are milked in stalls using a milking line, rather than being milked through the main herd milking parlour equipment (currently milking robots). This reduces the risk of cannulae being knocked out by other cows or by rubbing against farm gates/walls in a packed herd. The latter can still happen in a smaller group, but the risk is markedly reduced.

Food and water are withdrawn before surgery to reduce the amount of gut contents, thereby reducing the risk of inhalation of rumen contents during intubation and induction of

anaesthesia, and also reducing the risk of leakage of digesta during cannula placement. During surgeries that last longer than approximately 1.5 hours, a saline drip is infused into the jugular vein to reduce animal dehydration.

Following surgery, digesta contents may leak past the cannula through the fistula causing local skin irritation. Fistulation sites will be washed daily, and if any signs of skin irritation are apparent (e.g., dry/discoloured skin, sores or lesions, hair loss), they will be treated with a soothing ointment.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

Procedures requiring surgery will be carried out using aseptic techniques employed as recommended by the LASA 2017 Guiding Principles for Preparing for and Undertaking Aseptic Surgery (second edition).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Discussions with NACWOs and other PPL holders is an important part of ensuring that the latest and most appropriate methods in animal research are understood and used where possible. The 3Rs is a standing item on the organisation's AWERB agenda, which allows an open discussion at each meeting to ensure new methods of good practice are shared among all named persons. The NC3Rs has a monthly newsletter that provides updates on funding opportunities and publications, some of which may be relevant to the work and animal species that will be carried out and used under the authority of this project.

194. Investigation into the mechanisms causing acrodysostosis type 2 and related therapies

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Acrodysostosis Type 2, Skeletal dysplasia, Cognitive function, Hormone resistance, Therapy

Animal types	Life stages
Mice	adult, pregnant, juvenile, neonate, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of this project is to characterise a novel mouse model for the rare childhood and developmental disease Acrodysostosis Type 2 (ACRDYS 2). This disease is caused by a single change in a gene called phosphodiesterase-4 subtype D (PDE4D). This mouse model will allow us to better understand how mutations in the PDE4D gene leads to the different ACRDYS 2 symptoms in various tissues (e.g. brain, bone, fat) in the body and develop treatments that target specific tissues or the whole-body that can be brought forward to patients.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Acrodysostosis type 2 (ACRDYS 2) is a rare developmental disease where patients have a variety of symptoms such as small birth size, short height in adulthood, obesity, facial malformations, bone defects, reduced response to certain hormones and impaired brain functions. ACRDYS 2 is caused by genetic changes in the phosphodiesterase-4 subtype D (PDE4D) gene. The main role of PDE4D is directing the activity of key functions across several cells and organs throughout the body. In ACRDYS 2, the activity of PDE4D is abnormally increased. While the genetic cause of ACRDYS 2 is well understood, it is not clear how the increased activity of PDE4D leads to the range of symptoms observed in patients. Furthermore, there are several commercially available drugs that block the activity of PDE4D that could benefit ACRDYS 2 patients. Unfortunately, there are currently no animal models of the disease to allow these research questions to be correctly and thoroughly addressed. We therefore propose to characterise a novel mouse model for ACRDYS 2 that will carry the same genetic change found in ACRDYS 2 patients, termed S190A. This specific S190A change was chosen as it has previously been demonstrated to be defective in cell models and is located in a very important area that regulates its activity and function in cells. The animals will be observed to ensure that they have similar symptoms as ACRDYS 2 patients. This novel PDE4D S190A mouse model will be an invaluable tool for scientists, doctors and patients to help better understand the different components of the disease and importantly, develop new treatments that can prevent and/or slow down its progression.

What outputs do you think you will see at the end of this project?

SHORT-TERM:

- Characterisation of a novel ACRDYS 2 mouse model that will be made available to other researchers
- Evaluation of the therapeutic potential of commercially available PDE4D inhibitors
- Attendance and presentation of results at several national and international conferences
- Open access publications that adhere to ARRIVE guidelines

LONG-TERM:

- Mechanistic insights into ACRDYS 2 pathophysiology
- Development of new treatments
- New collaborations with clinical teams and industrial partners
- Acquisition of follow-up funding

Who or what will benefit from these outputs, and how?

This new transgenic mouse model will directly contribute to fundamental researchers and clinicians in the ACRDYS 2 field, providing a valuable pre-clinical model to better understand the pathophysiology of the condition and evaluate the potential of drug repurposing strategies. In addition, this model will be beneficial to academics and clinicians in the larger field of pseudohypoparathyroidism (PHP) and related disorders (e.g. PHP type 1A, PHP type 1B, pseudopseudohypoparathyroidism (PPHP), progressive osseous heteroplasia (POH) and ACRDYS 1), allowing the deciphering of pathological similarities and distinctions between each condition and providing an additional resource to evaluate potential treatments across a family of related conditions.

Interestingly, there has been a recent interest in the PDE4D gene as it has been implicated in Alzheimer’s disease and general cognitive performance. Thus, once we have completed the preliminary assessment of cognitive (dys)function in this transgenic mouse model, we believe that it will generate great interest from fundamental researchers and clinicians in the ageing and neuroscience fields alike and be used for further mechanistic and therapeutic investigations.

Finally, as these transgenic mice are predicted to display a range of distinct symptoms, we anticipate that they will also be useful to academics and clinicians in a number of research fields such as bone development and growth, endocrinology and metabolism.

Thus, by characterising a novel mouse model of ACRDYS 2, we will also be contributing to wider national and international scientific, clinical and public communities.

How will you look to maximise the outputs of this work?

Dissemination of results will be achieved by presenting our work at national and international conferences, publishing in open access journals and through wider public engagement endeavors (e.g. general awareness events, academic profile page, professional Twitter account). Furthermore, our collaboration with the patient organisations will provide multiple opportunities to share our work with the Acrodysostosis community.

Species and numbers of animals expected to be used

- Mice: A maximum of 1425 mice is estimated to be used during this 5-year project, which includes breeders and experimental mice (genetically modified and wild type).

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To understand how ACRDYS 2-causing mutations in the PDE4D gene lead to the whole-body presentation of ACRDYS 2 over the life course and to evaluate the impact of

therapeutic interventions on these symptoms during disease progression, a whole-body complex system such as a representative mouse model is required.

Typically, what will be done to an animal used in your project?

Typically, an animal will be aged to 3, 6 and/or 12 months, be weighed weekly and undergo a learning and memory test. A small number of animals will also be given a drug by daily injections.

What are the expected impacts and/or adverse effects for the animals during your project?

The adverse effects expected are based on the manifestation of this rare disease in patients: bone malformations, underdevelopment of facial bones, learning disabilities, delays in motor and coordination abilities, impaired response to hormones, obesity and delayed growth and development (at birth and subsequently). These symptoms occur equally in males and females and are either present at birth or in the early stages of life. With early diagnosis and interventions, children with Acrodysostosis Type 2 can reach their full potential and expect a normal life expectancy. We therefore expect the novel transgenic mice to have a smaller birth weight, a delayed growth spurt, shorter or malformed bones, impaired learning and memory abilities throughout life and increased weight gain over time. In addition to these adverse symptoms directly related to the PDE4D S190A mutation, brief instances of pain may also be felt during drug injections. All adverse effects are minimised by very close monitoring of mice with designated score sheets and selecting appropriate humane endpoints.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Subthreshold: 5%
- Mild: 20%
- Moderate: 75%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

There is a scientific need for an ACRDYS 2 mouse model to inform on how the disease starts and how it progresses as well as evaluate and develop new treatments. As ACRDYS 2 affects several organs and systems across the whole-body, a whole biological system is required to optimally investigate how different tissues and organs act individually and together to cause the disease.

Which non-animal alternatives did you consider for use in this project?

Non-animal alternatives such as mathematical modelling, cells and tissues were considered and not deemed appropriate to achieve the aim of this project.

Why were they not suitable?

To understand how ACRDYS 2-causing mutations in the PDE4D gene lead to the whole-body presentation of ACRDYS 2 over the life course and to evaluate the impact of therapeutic interventions on these symptoms during disease progression, a whole-body complex system such as a representative mouse model is required.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

Using commonly used and approved software programmes and guidelines such as the NC3Rs Experimental Design Assistant, ARRIVE Guidelines 2.0 and G*Power software as well as previous relevant experience with similar studies, both from our research group and published by others. Power calculations were based on previous literature.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Using commonly used and approved software programmes and guidelines such as the NC3Rs Experimental Design Assistant, ARRIVE Guidelines 2.0 and G*Power software. Power calculations were based on previous literature .

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

- Efficient breeding strategy to produce the maximal number of experimental mice of the desired genotype (genetically modified and wild type).
- Use of both females and males.
- Use of control cohorts across several analyses.

- Collection of blood and multiple tissues from one single animal for multiple analyses.
- Use of the same animal to undergo the same memory and learning test at different ages.
- Tissue sharing (if appropriate).
- Constant review of results to amend experimental plan if necessary.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

- We will be using genetically modified mice that harbor a single mutation in the PDE4D gene (PDE4D S190A) to produce a model of ACRDYS 2.
- We will also use non-genetically modified animals.
- The memory and learning and motor function tests are commonly used and they have been designed to provide scientifically valid data whilst minimising exposure to pain, suffering, distress or lasting harm to the animals.
- In instances where drugs are administered, they will be administered via a brief prick to minimise pain and lasting harm to animals.

Why can’t you use animals that are less sentient?

We have considered less sentient animals such as fish, worms and flies but these are inadequate for the successful achievement of the aims of our project as:

- There is a scientific need for a mammalian animal model of ACRDYS 2 that closely resembles the human disease.
- Different ages (e.g. juvenile, adult, aged) are required to assess the progression of whole-body symptoms during the life course of the animal.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

- Undertake an initial study with only a small number of animals to begin with. A larger study with more animals will only be undertaken following discussions with expert personnel (e.g. veterinarian).
- Review and adapt experimental plan (e.g. ages used) as data in earlier ages are analysed.
- Use score sheets to monitor well-being of aging animals or those undergoing memory and learning tests. The score sheets will be developed and reviewed by expert personnel (e.g. veterinarian).
- Have a habituation session for animals undergoing memory and learning tests to allow them to adapt and familiarise themselves with the test.

- Add padding to elevated tests (e.g. rotarod) to avoid injury.
- Place animals in a warming chamber to keep them warm after the memory and learning tests.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to the resources provided on the NC3Rs website and the ARRIVE Guidelines 2.0.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

- By accessing the information on the NC3Rs website: https://nc3rs.org.uk/experimental-design
- By participating in relevant workshops.
- By updating the NC3Rs Experimental Design Assistant diagrams as the project progresses.

195. Neural mechanisms of visual cortical plasticity

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

vision, rodent, cortical plasticity, imaging, electrophysiology

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant, embryo

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The goal of this project is to reveal key cellular and circuit mechanisms employed by the adult brain to adapt and change in response to natural and artificial experience. We will combine genetic manipulation with chronic imaging / electrophysiology to study various forms of neuroplasticity in the intact adult animals, at synaptic, cellular, and circuit level.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?
The limited plasticity in the adult brain is responsible for reduced visual learning ability and incomplete vision recovery from injury, diseases, and ageing. The successful completion of this project will uncover important biological principles governing plasticity that can be generalised to other visual pathways and higher species, providing us a mechanistic framework for potential therapeutic interventions to promote rehabilitation and visual perceptual learning in adult human patients.

What outputs do you think you will see at the end of this project?

Completion of these objectives will provide a biological basis how visual plasticity in the adult brain can be achieved through interactions within local cortex and interaction with other brain areas. In the process, we may uncover important principles governing plasticity that can be generalised to other cortical areas and in higher species.

Who or what will benefit from these outputs, and how?

In the short-term, the research output is of direct interest to neuroscientists in the field of sensory development and plasticity. The brain maintains a lifelong capacity to undergo structural and physiological changes, which was once thought to happen only in the developing brain. This project will reveal key cellular and circuit mechanisms employed by the adult brain to maintain this capacity, especially emphasising on the less studied interneurons and neuromodulatory pathways.

In addition, the physiological and anatomical results in vivo and ex vivo will be useful for comparative analysis, enabling the field to form a comprehensive view of cortical plasticity and promoting the computational modelling and ex vivo research that can replace in vivo work.

In the long run, our knowledge in neuroplasticity might contribute to translational research for designing mechanism-based therapies for cortical functional recovery. For example, understanding the process of cortical plasticity resulting from electrical stimulation is critical for optimal design of stimulation-based therapies in treating human patients with malfunctional brain.

How will you look to maximise the outputs of this work?

Our scientific findings and other resources will be provided to broad communities through publications and presentations. We will also partner with bioengineers and clinical

scientists to promote translational research focusing on enhancement of brain function or recovery from disease and injuries.

Species and numbers of animals expected to be used

- Mice: 3000

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Mice are used as the animal model in the proposed project to study visual cortical functions and plasticity. This choice is in line with prevalent opinions in the past decades, due to 3 major reasons.

First, rodent visual system shares important similarities with that of larger mammals, like carnivores and primates. For example, functional and anatomical evidence confirmed that visual information in mice is also streamed in ventral-like and dorsal-like pathways, together with a complexification of the processed features along the hierarchy. These across-species preserved architectures suggest fundamental commonalities in the underlying computations.

Furthermore, the accessibility of increasingly powerful experimental approaches in mice, including transgenic manipulation, optogenetics, and two-photon imaging, that makes it an ideal model to dissect the underlying neural circuits.

Finally, rodent models also offer great potential to explore behavioural and cognitive processes that rely on vision, including visual perception, vision-guided choice, and vision-innate behaviour link.

Typically, what will be done to an animal used in your project?

Wild type and genetically altered (GA) mice will be bred, genotyped, and maintained. Animals will go through surgical procedures to allow chronic imaging/recording over days (no water restriction, usually 1-2 hours each day, for a few days to months). After recording in intact animals either in awake behaving states or under anaesthesia, the brain issue might be collected for ex vivo anatomical and/or physiological characterization for comparative analysis.

What are the expected impacts and/or adverse effects for the animals during your project?

To allow access to the brain via optical or electronic means, the mice undergo surgical procedures. During these procedures they are fully anaesthetised and they do not experience pain. Effective analgesia is provided after each surgery. Use of sterile techniques, strict analgesic regimes and careful monitoring of the animals during and after the surgery minimises any potential pain or distress experienced by the animals.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Moderate severities are expected for the experimental animals.

What will happen to animals at the end of this project?

Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Our experiments aim to understand the long-term of brain activities that result from natural and/or artificial experiences. Therefore, we will perform experiment in the intact animal, and in various behavioural states (running, still, anesthetized).

Which non-animal alternatives did you consider for use in this project?

In vitro preparations and neuron network modelling are considered to study the neuronal circuit plasticity as non-animal alternative.

Why were they not suitable?

Sole in vitro preparations are unable to answer the proposed answers, because 1) we want to understand the long-term (days to weeks) changes of neural network activities in intact animals; 2) we aim to study how animal's behavioural states (running or still) change affect this process. However, we will keep track of research literature about specific 2D/3D cortical organoid model in the field to seek appropriate alternatives. We also propose to conduct research using in vitro preparation after in vivo measurement to enhance the general scientific conclusions and build links between the two systems, providing a basis for the replacement in the future studies.

Computer modelling requires the in-depth knowledge of the connections between cells and visual function. The parameters for modelling needed to constrain such simulations are currently unclear. In fact, a side benefit of our research is to provide some of these parameters to enable in vitro preparations and computational studies in the future.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We aim to use the minimal number of animals consistent with previous knowledge and experience working in the field of visual neuroscience. We estimate the total numbers of

the animals based on the transgenic breeding, successful rate of the surgical procedures, and completion rate of the trainings.

The historical and published data for all the techniques named in this project also provide an estimation of variance for many planned experiments, which allows us to decide the number of animals required to provide robust experimental results using statistical power calculations.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The experiments are carefully planned in order to minimise the number of animals needed to obtain enough data to achieve the aims of the different projects.

Experiments will be designed according to the NC3Rs ARRIVE Guidelines to ensure experimental rigour (blinding, randomisation etc) whenever possible. When necessary, we will also seek for statistical advice from professional services, such as the NC3R's Experimental Design Assistant https://eda.nc3rs.org.uk/guide.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

First, we will seek for advice on breeding transgenic lines, to avoid unnecessary production of animals. When possible, we will share common transgenic lines with other laboratories, instead of breeding them ourselves. Depending on the breeding performance of specific strains, we will strive to use intermittent breeding instead of constant mating to avoid unnecessary wastage of animals, following advice from https://nc3rs.org.uk/worked-example-intermittent-breeding.

In addition, data previously taken will be used to answer new question and we will resort to available databases (e.g. NeuroData without borders: http://www.nwb.org/allen-cell-types-database/).

Third, we have perfected techniques to record from vast numbers of neurons simultaneously and chronically. We will image/record the same population of hundreds of neurons using miniscope / 2- photon imaging / electrodes, and the animals will be tested for periods of several days to weeks and thus considerable information is obtained from each animal, minimising the total number used.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project?

Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

The mouse is the species of choice because rodent visual system shares important similarities with that of larger mammals, and transgenic strains allow for multiple measurement/ manipulation.

Chronic optical/electric recordings in mice cause minimal pain / suffering, moreover, because they are either in an awake behaving state (head-fixed to run on a 2D/3D treadmill or freely moving), or in a well- maintained anesthetized state.

Why can't you use animals that are less sentient?

We need to study brain plasticity in the adult animals because the adult brain is less capable of change and adaptation and mechanistically different from juvenile animals. There is an urgent need to understand neural basis and find ways to promote plasticity in the adult brain. Our project will address aspects of this.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

We will minimise pain and discomfort during and after all the procedures performed with animals:

Surgeries will be performed under general anaesthesia. Analgesics and antibiotics will be administered as needed during and after surgery to minimise pain and reduce the risk of infection, respectively. The surgeries will be performed by fully trained and competent staff to minimise side effects and the length of the surgery. All animals will receive post-operative care to ensure a normal recovery. If any animal fails to recover from the surgery, it will be humanely euthanized by a Schedule One Method immediately.

To minimise discomfort after surgery, we will use low-weight head-plate and brain implants (the head- plate is less than 1gram, and in some cases with additional implant but less than 10% of the animal's body weight), so that the mice can move normally in their home cages and have minimized brain insults. And most of our techniques in subsequent experiments are minimally invasive, as they involve imaging through the skull or through a cranial window. If being head-fixed in a test/training rig, mice will be progressively habituated to the behavioural rigs so that they are not scared of spending time there.

Mice are cohoused wherever possible. Stress during experiment will be minimised by gently handling the animals with refined methods (e.g. cupping), and by getting them used to the experimenter. Where head restraint is required to avoid animal movement, distress will be minimised by gradual habituation– for example, 15 mins on the first day, 30 mins on the second day, and 45-60 mins by the third day.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will use the webinar offered by NC3Rs as a guidance to refined approach to our procedures (https://www.nc3rs.org.uk/events/nc3rs-webinar-refining-rodent-stereotactic-surgeries).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

We will get refresher training promptly and consult with Named ASPA personnel, i.e. NIO , NVS, NACWO, etc to keep up with the lasted information in the 3Rs. We will also follow up regularly with the NC3Rs advocate in our institute, who regularly disseminates new information about experiment design, methods and replacement technologies to all investigators involved in animal research.

196. Immune and inflammatory mechanisms of cardiovascular disease

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Atherosclerosis, Cardiovascular disease (CVD), Hypertension, Immune response, Inflammation

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged
Rats	adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The main objective of this research program is to define the critical turning points of processes leading to the overactivation of immune defences in diseases of blood vessels and high blood pressure.

Through proposed research, we will address one of the most important unmet clinical needs in cardiology. We will identify new and improved treatments for plaque build-up in arteries (atherosclerosis) and high blood pressure.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these

could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

High blood pressure and dysfunction of blood vessels are causing at least 20 million deaths/year, exceeding any underlying cause, including the recent coronavirus pandemic.

Work carried out in this project will identify the essential interactions in cells and particles responsible for body defence from bacteria and viruses (immune system) underlying the initiation and development of cardiovascular disease leading to heart attack and stroke. These studies will show how, when and where these interactions occur between different cell types, which drive overactivation of body immune defences in high blood pressure and associated cardiovascular diseases. This information is fundamental as it will reveal new ways we could use to treat these diseases using newly developed drugs.

Large international studies in patients are recently starting to demonstrate the utility of medications affecting body immune defences in diseases of vessels and the heart; however, none of these medications is currently used in standard care. The current proposal will identify the best ways to modulate body immune defences in the heart and vessel pathologies. Based on this, we will be able to design better medications, modify existing drugs, and apply them more rationally in patients with high blood pressure and heart and vessel diseases. This will bring benefits to patients in areas where there is an unmet clinical need.

What outputs do you think you will see at the end of this project?

Outputs can be considered on several levels: New scientific knowledge regarding mechanisms of overactivation of immune body defences in diseases of blood vessels. The aim is to publish findings in academic journals, disseminate them promptly to the scientific community and translate them to clinical impact. We will present data to our scientific colleagues at conferences, other universities, researchers within pharmaceutical companies, clinicians, and the lay public.

The immediate outcome will add to the knowledge of what causes heart attacks and high blood pressure. Based on this knowledge, rational design of new medications will be possible to target body immune defences. This would be of enormous potential benefit to human health, animal welfare and the economy.

Who or what will benefit from these outputs, and how?

Scientists - New scientific knowledge regarding mechanisms of overactivation of immune body defences in diseases of blood vessels.

Society – Discovery of new methods to treat high blood pressure or plaque build-up in blood vessels will bring potential to improve the health and quality of life of millions of patients worldwide.

Economic benefits – Reduction of the burden of cardiovascular disease will result in huge savings of health systems worldwide. Thanks to more advanced methods, the therapies proposed will be more effective and better tailored to the needs of patients. This will make them more financially feasible for the pharmaceutical industry to provide to patients.

How will you look to maximise the outputs of this work?

These studies would provide needed information to clinicians and scientists involved in research understanding the role of body immune defences in heart and vessel diseases. We aim to publish novel findings in the highest quality journals. We consider this as the primary medium for the communication of the outcomes from this research. Publications will be made open access and presented at national and international conferences, policy briefs, and social media. Critical data obtained will be shared freely with the scientific community by posting on data repositories.

Finally, we are strongly committed to outreach and science communication. In the last three years, we have been leading the organisation and delivering an exhibit at major science festivals interacting face-to-face or online with more than 60,000 people. We routinely communicate scientific advances to the public via social media.

Species and numbers of animals expected to be used

- Mice: 9500

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We propose to use mice, as the smallest animals on the evolutionary scale, in which suitable models are available to use in studies of the role of overactivated immune responses in cardiovascular disease. Furthermore, the genetically modified models that we propose to use have only been developed in mice. Adult mice will be used because the development of cardiovascular disease studied occurs at the adult stage of life.

Typically, what will be done to an animal used in your project?

The animals will be subjected to the factors leading to high blood pressure and vascular disease. This will involve the administration of substances that increase blood pressure or circulating levels of cholesterol. Body immune defences will be changed through the administration of specific drugs or molecules via different routes (e.g. intraperitoneal,

intravenous, and subcutaneous under the skin or using specific delivery osmotic minipump). In some cases, transfer of blood forming cells preceded by irradiation is needed to provide a long lasting and specific change in body immune defenses. Mouse models of high blood pressure are advantageous as the duration of experiments is generally 14 days. We will carefully monitor blood pressure throughout the experiment using either tail-cuff. The animals will then be killed for analysis of their blood vessels, kidneys and other organs. Models of plaque build up inside the arteries are evoked by feeding animals a high-fat diet for usually at least three months.

The animals will then be killed for post-mortem measurements of plaque build-up inside the arteries.

What are the expected impacts and/or adverse effects for the animals during your project?

Protocols are subthreshold, mild or moderate. The animals may experience different adverse effects depending on the protocol. For example, weight loss and diarrhoea following irradiation, skin irritation following feeding with a high-fat diet may be observed. The expected adverse effects are transient with estimated times between maximally 48 hours (diarrhea, skin irritation) – 2 weeks (weight loss; skin irritation on HFD).

Anesthetised and conscious animals will be used in these studies, and surgical techniques will be kept to a minimum. Any surgical techniques will be carried out under general anaesthesia. Analgesia will be used.

In the end, animals will be euthanised.

We will closely monitor all animals. Any animal displaying deviation from normal health, other than due to the inevitable effects of the procedure, will be promptly killed or withdrawn from the procedure and referred for veterinary attention. We will keep the procedures to the minimum duration possible.

For each protocol, specific monitoring systems will be used. These will be reassessed and reviewed throughout the study. Detailed and up-to-date guidance will be monitored and followed via the NC3Rs website (http://www.nc3rs.org.uk/).

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

- Sub-threshold - 2000 mice - 21%
- Mild - 3000 mice - 31%
- Moderate - 4500 mice - 48%

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Cardiovascular disease and inflammation result from a complex interplay of environmental and genetic factors on multiple target organs. Such mechanisms are impossible to be adequately reproduced in the cell culture models or using computer modelling. Therefore, further work in living animals is required to address these questions. We will continue to carefully observe a broad range of portals and websites (e.g. FRAME, CAAT-Europe, NC3Rs) to keep abreast of the development of new and valid methods that could replace the need for laboratory animals in medical and scientific research. These resources offer no specific alternatives at present.

Which non-animal alternatives did you consider for use in this project?

Studies in cultured cells and isolated human blood vessels will be employed where possible, having a variety of collaborators with whom we regularly discuss the potential options for replacement.

Why were they not suitable?

These models are suitable to address few selective questions. Still, they cannot fully reproduce the interplay of multiple environmental and genetic factors that affect numerous target organs in cardiovascular disease in animal models. Thus, alternative approaches can provide correlative type data, and powerful cause-effect reasoning requires the use of animals.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

A balance between keeping animal numbers to a minimum and achieving statistical validity in experiments has to be achieved. Previous extensive experience has enabled us to ensure that animal usage in our studies is kept to a minimum. Each experiment is carefully

designed to provide the minimum use of animals necessary to achieve our scientific aims. We have estimated to use around 9,500 mice in the five years of the licence. This will include 5,000 mice within the breeding and maintenance; 500 mice for irradiation protocol and 2,000 mice for atherosclerosis and 2,000 mice for hypertension protocol. The balance between these numbers is related to the balance of ongoing projects we have been conducting in each of these areas.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The statistical framework for our experiments has been developed with advice from a statistician. Each protocol is based on extensive experience and is optimised to ensure minimal suffering for the animals involved. We routinely use the sample size calculator provided in the Experimental Design Assistant (EDA) to determine study sample sizes. We will use appropriate study designs. For example, we will use factorial design when studying the effects of pro-hypertensive substances in male and female mice. We will also use block randomisation when appropriate.

We minimise possible unintended error and data distortions (bias) by directly and objectively measuring the properties of blood vessels, cholesterol and blood pressure. Subjective scoring by investigators will only be used where objective methods are not available. Mice will be randomly assigned to groups by staff blind to experimental details using dedicated software (https://www.r- project.org/).

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use whole-body imaging systems to monitor disease onset in a minimally invasive fashion. Thus, repeated imaging of the same animal can be carried out to improve our data quality and reduce the number of animals required for time-dependent studies.

To further reduce the need for extensive backcrossing of transgenic and knockout animals, we will use a recently developed method to induce plaque build-up in blood vessels by a single vector injection.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Mice are used in this project as they represent the least developed small animal group where models of cardiovascular disease directly relevant to the human pathology can be developed. At the same time, there is an extensive understanding of the immune/inflammatory system of the mouse. Moreover, several genetically modified models are only available in mice.

We propose to use established models of high blood pressure and arterial plaque build-up, corresponding to properties of human cardiovascular disease. The protocols have been chosen to provide maximally detailed information while ensuring that the animals under investigation experience the lowest possible pain, suffering, distress, or lasting harm. For example, the administration of substances will be carried out by the least severe/painful method available. Animals will always be acclimatized to handling within each procedure. For metabolic cage studies, animals will always be housed in pairs or groups unless individual housing is required due to welfare reasons or for scientific purposes. We usually use in-house modified metabolic cages that have tinted walls and a solid platform so that animals feel more secure and comfortable. Moreover, experiments will be carried out for short periods (e.g. overnight). For blood pressure measurements we will limit the use of invasive telemetry to only selected animals/experiments, as it requires surgical telemeter implantation and will use tail-cuff in more routine experiments. The use of telemetry is required to establish a fully objective measure of blood pressure changes in similarity to 24-hour blood pressure monitoring in humans that is required for diagnosis.

Animals bought from accredited breeders are always given a period of acclimatization and handling before use experimentally. Our staff always uses delicate handling. Mice are lifted using a tunnel rather than by their tail. This can lower their anxiety, enhance welfare, and increase the chances of obtaining more precise research results. Our models of cardiovascular disease have been developed and refined and give repeatable results of low variability and high quality. Each protocol is based on extensive experience and has been optimized to ensure minimal suffering for the animals involved. Throughout, we will ensure that we apply the least invasive methods of dosing and sampling. We will carry out surgical procedures aseptically, using an anaesthesia during procedures and analgesia to control post-operative pain. When multiple subcutaneous injections are called for, we will use osmotic mini-pumps to minimize suffering wherever appropriate. Where procedures are known to cause adverse effects, we will initially use the method using the least severe adverse effect. Animals will be given soft bedding and soft food to alleviate suffering where appropriate.

The humane endpoints have been established so that veterinary opinion should not be required before animals are killed. However, any animal displaying deviation from normal health, other than due to the inevitable effects of the procedure, will be promptly euthanized or withdrawn from the procedure and referred for veterinary attention. The procedures will be kept to the minimum duration possible.

For each protocol, we will use specific monitoring and scoring systems. These will be reassessed and reviewed throughout the study. Up to date, we will carefully monitor guidance via the NC3Rs website (http://www.nc3rs.org.uk/).

Why can't you use animals that are less sentient?

We cannot use animals that are less sentient because, for both high blood pressure and plaque development inside the arteries, the mouse is the least sentient species allowing to reproduce cardiovascular features relevant for human pathology. Plaque buildup assessment and blood pressure outcomes cannot be relevantly measured in less sentient animals. We use adult mice as atherosclerosis and hypertension are primarily diseases of adulthood. In addition, cardiovascular diseases are chronic disorders that cannot be studied in terminally anaesthetised mice.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Our models of cardiovascular disease have been developed and refined and give repeatable results of low variability and high quality. Many of the models and methods to be employed have been refined to reduce suffering to the minimum levels likely to give satisfactory results. Animals will be given soft bedding and soft food to alleviate suffering where appropriate. Analgesia is used for all procedures that may cause pain.

We are constantly performing refinement of our procedures.

Whenever alternative possibly better methods/models appear we aim to perform and publish refinement work in similarity to what was described above.

The humane endpoints described have been established such that veterinary opinion should not be required before animals are euthanized. However, any animal displaying deviation from normal health, other than due to the inevitable effects of the procedure, will be promptly euthanised or withdrawn from the procedure and referred for veterinary attention. The procedures will be kept to the minimum duration possible.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will be following the ARRIVE guidelines to ensure good laboratory practice and transparent scientific reporting (e.g. randomisation and blinding to reduce bias, power calculations to avoid Type I and II errors and integration of the 3Rs).

Experiments on experimental plaque development in arteries as well as high blood pressure and hypertension will be performed in line with the most recent recommendations on the design, execution, and reporting of studies in animal models published by the American Heart Association.

Up to date guidance will be monitored via the NC3Rs website (http://www.nc3rs.org.uk/).

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

Up to date guidance will be monitored via the NC3Rs website (http://www.nc3rs.org.uk/). In addition, we will follow internal 3Rs activity and communication (such as Culture of Care meetings, 3Rs days, etc.).

197. The aberystwyth liver fluke sample-bank for the evaluation of diagnostic tests

Project duration

5 years 0 months

Project purpose

- Translational or applied research with one of the following aims:
  - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants

Key words

Liver fluke, Rumen fluke, Diagnostic tests, Sheep

Animal types	Life stages
Sheep	juvenile, adult

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

To test the ability of two new diagnostic tests to differentiate between sheep that are infected and those that are not infected with liver fluke (Fasciola hepatica). The samples that are collected will also be used to create a bank of blood-sera samples which will be available to evaluate further liver fluke diagnostic tests in the future.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Liver fluke (Fasciola hepatica) is a trematode parasite with significant effects on human health and currently infects around 2.4 million people worldwide. It is also a parasite with extensive impacts on the livestock industry and is estimated to cost €2.5 billion globally per

year, mainly due to impacts on animal health and food security. The UK Government's recently published Life Sciences Vision (https://www.gov.uk/government/publications/life-sciences-vision) highlights the strategic importance of the Life Sciences sector and emphasises the need to grow capacity in translating basic scientific research to real world applications. This work will be key in delivering the next crucial steps to develop novel diagnostic tests for liver fluke.

The 'Gold Standard' method for identifying liver fluke infection (fascioliasis) is by conducting a post- mortem and examining the liver and bile ducts of animals for the presence of the parasite or visible damage to the organs. There is currently no 'Gold Standard' diagnostic test that can be used to diagnose fascioliasis in live animals. This is a major animal health and welfare issue as accurate, affordable, and readily available diagnostic tests are key to the ability of farmers and veterinarians to administer appropriate treatments to livestock. The lack of accurate diagnosis prior to treatment is seen as a significant contributor to the growing threat of resistance to key therapeutic chemicals (anthelmintics) which threatens the future control of liver fluke. This project will compile a sample bank that will be able to play a significant role in determining the ability of new diagnostic tests to correctly diagnose liver fluke infection in sheep.

What outputs do you think you will see at the end of this project?

The immediate aim is to use the sample-bank to establish the performance of two new liver fluke diagnostic tests. We expect to publish the outputs in peer-reviewed journals which will showcase the performance of these tests in the literature. This will be a key step in allowing the consortiums involved with developing the diagnostic tests to deliver the tests to end users (such as farmers and veterinarians). The long-term aim is to use the samples gathered as part of the project to create a sample-bank that will be a long-term resource for establishing the performance of further liver fluke diagnostic tests.

Who or what will benefit from these outputs, and how?

In the short-term, the project will allow us to establish the performance of two new fluke diagnostic tests with naturally infected sheep (1: the adaptation of the FECPAK G2 faecal egg counting system for use liver fluke; 2: FlukEVal sera based liver fluke ELISA). This approach is considered a necessity as it reflects the situation (naturally infected farm livestock) for which the test will ultimately be used by end users. This is a necessary step in the development of any veterinary diagnostic test and will be a key milestone in bringing these tests to the marketplace and for use by end users (farmers and veterinarians). Making these tests available to end users has numerous potential benefits. Better availability and choice of fluke diagnostic tests will make diagnosing fasciolosis more accessible to livestock keepers. This will bring welfare benefits to livestock as liver fluke has been shown to have notable negative welfare effects. Correct diagnosis is also a key factor in reducing over-use of key flukicide anthelmintics to reduce the development of resistance which risks undermining the long-term control of these parasites. More effective

parasite control can also bring farm business benefits through lower costs and more efficient production as well as potentially reducing farmer mental health strains.

In the longer term, our aim is to create a sample-bank that will allow further liver fluke diagnostic tests to be evaluated on samples from naturally infected sheep without the need for further sheep to be used.

This will establish a legacy for the project and bring 3Rs welfare benefits, by reducing the need for developers of future novel diagnostic tests to undertake regulated procedures on sheep in order to gain samples to validate their tests.

Information on the disease status of the sheep in the project will be fed back to the stock keeper and they will be free to use that information to inform appropriate treatments for their animals.

How will you look to maximise the outputs of this work?

This project will create a sample-bank that will be used beyond the lifetime of this project. For example, the sample-bank will be used to evaluate the performance of further diagnostic tests beyond those identified in this project. We will actively encourage other users to use the sample-bank through formal collaborations and licencing of the sample-bank. This will provide external institutions an opportunity to undertake evaluation of novel diagnostic liver fluke tests without the need to undertake further sampling in sheep which has considerable potential for long-term 3Rs benefits. We aim and expect to gather up to 10 ml of blood which should lead to at least 1 ml of sera per sample. We expect that each 1 ml of blood sera obtained will be able to be used in 100 separate reactions. This will provide a resource that will be available for a considerable amount of further work.

Species and numbers of animals expected to be used

- Sheep: 300

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

Liver fluke (Fasciola hepatica) is an internal parasite which infects livestock through grazing infected herbage. Consequently, it only infects sheep which naturally graze infected pasture. This precludes the use of foetal life stages and very young lambs which have not yet started grazing herbage and thus have not had the opportunity to become naturally infected with liver fluke. Juvenile lambs and adult sheep are the most appropriate life stages to use for the project as they will have been given opportunities to be challenged by fluke infections. This will also allow us to quantify performance for animals that will be in their first grazing season and those who may have been infected previously.

Typically, what will be done to an animal used in your project?

Sheep on selected commercial farms with high liver fluke prevalence will be selected for inclusion in the project with the consent of the owners. Suitable sheep from these flocks will be identified via their ear tags and samples of faeces will be collected by rectal grab (or from floor droppings if possible).

Blood will also be collected from the jugular vein of each sheep. The sheep will be held in a holding pen and each farm's vet will be asked to verify that the animals are not in pain, suffering, or distress or likely to have any lasting harm as a result of the procedures undertaken. Sheep will be inspected on the day the procedures are undertaken and they will be released back into the care of their owner after confirmation from the vet. This will end their role in the project.

What are the expected impacts and/or adverse effects for the animals during your project?

The expected impacts and/or adverse effects of the two regulated procedures are expected to be mild and short lived. This may include slight discomfort during the procedures, but discomfort should stop shortly after completing the procedures.

The cumulative effects of two regulated procedures in quick succession is worth noting but undertaking the procedures in a competent manner should still ensure that the expected impacts and/or adverse effects are minimal/very mild.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

The severity level is expected to be 'mild' in all cases and for all animals in the project.

What will happen to animals at the end of this project?

- Kept alive

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Diagnostic tests that aim to identify whether sheep are infected with parasites such as liver fluke need to be able to demonstrate a suitable ability to differentiate between infected and non-infected animals within flocks in order to fulfil a useful role for farmers and

veterinarians, and in order to aid animal welfare through correct diagnosis that leads to appropriate treatment.

Sheep in commercial flocks that are infected with parasites such as liver fluke are exposed to different naturally occurring parasite populations at different locations on farms and differences in grazing patterns between individuals, which will lead to some sheep becoming heavily infected whilst others are less heavily infected. At the same time, sheep in commercial flocks will also be challenged by other naturally occurring co-infections such as rumen fluke, parasitic nematodes and other diseases and parasites which can affect a sheep's immune system and potentially have a hidden effect on diagnostic tests.

These factors and are main reasons why evaluating diagnostic tests in naturally infected populations that resemble the conditions on commercial farms is considered necessary in order to provide a suitable evaluation of their effectiveness value for farmers and veterinarians and why we need to use animals to achieve the aim of our project.

Which non-animal alternatives did you consider for use in this project?

Possible alternatives to using live animals in this project include the use of samples gathered from sheep slaughtered in commercial slaughterhouses. These could be naturally infected samples that are used directly to evaluate the diagnostic tests or samples that test negative for liver fluke to which we then add a known quantity of suitable parasite life stages prior to use.

Why were they not suitable?

Evaluating the performance of diagnostic tests with samples where a known quantity of suitable parasite life stages is added is an appropriate step in the evaluation process. This is a step we have already undertaken for one of the novel diagnostic tests we wish to evaluate with the sample-bank. However, this does not provide an estimation of the performance of the diagnostic test with naturally infected animals and does not represent the conditions which the test will be used by farmers or veterinarians and therefore is not suitable as a definitive final estimation of the performance of diagnostic tests for liver fluke.

Gathering samples from sheep slaughtered in commercial slaughterhouses does provide an opportunity to gather samples from naturally infected carcasses immediately after slaughter but our previous experience has shown us that the practical limitations of processing samples in a slaughterhouse setting means that it is not possible to obtain a high enough liver fluke prevalence. For example, practical limitations mean samples must be stratified between groups which originate from different farms and which are likely to have very different fluke prevalence levels due to the speed of carcasses moving through the abattoir). This means that it is impossible to devise a practical method to sample intensely from high liver fluke prevalence groups. Furthermore, abattoirs are high risk for SARS- CoV-2 transmission between personnel, and their operation is highly dependent on a large workforce.

This has led to many abattoirs restricting non-staff to enter the premises for the foreseeable future.

Obtaining samples from groups of sheep with both low and high fluke prevalence is a necessity in order to provide an effective estimate of diagnostic test performance in a Bayesian Latent Class statistical framework in the absence of Gold standard comparator tests. We have concluded that the only practical way of obtaining samples from groups of sheep with fluke high prevalence levels is to sample sheep on farms purposely selected for their high fluke prevalence levels.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have used an appropriate sample size estimation method that can use expected diagnostic test sensitivity and specificity measures to estimate the numbers of animals needed. The method can make use of initial estimates of test sensitivity and specificity and these are sourced from data already gathered, including pilot data. The data we already hold has been used to reduce the numbers of animals needed through refining our strategy for selecting the commercial farms where we will source the animals. In effect, we already possess enough samples from animals that are not infected with liver fluke. This allows us to optimise animal numbers by using a strategy to increase the proportion of fluke positive sheep in the animals we will use as part of the project (and thus minimising the number of fluke negative sheep we use in the project).

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

The framework for evaluating diagnostic tests assumes that each sample is independent and this precludes the use of duplicate samples from the same animal. However, we already have 500 samples (~40-50 fluke positive and ~450-460 fluke negative) from a previous abattoir-based study that is part of the sample-bank. This means that the sample-bank already includes enough negative samples to establish the specificity (the ability of the test to correctly identify those without the disease) of the diagnostic tests that the project will evaluate initially and for longer-term use in the sample bank.

Consequently, the project’s efforts can be focused on sampling sheep from farms that are most likely to be infected with liver fluke. As the infection status of individual sheep will not definitively be known prior to sampling, the total number of sheep that will need to sampled will be influenced by the prevalence of the parasites in the overall sheep population used

in the project. We can leverage the fact that the project only needs to gather samples from infected animals to select farms likely to have the highest prevalence and to reduce the total number of sheep to sample.

On-farm prevalence of liver fluke is determined by a range of factors that includes the history of fluke infections and treatments within individual flocks and consequently some farms and groups of sheep within flocks that have been grazing high risk pastures can have very high prevalence levels above 70- 80%. Fluke prevalence is also strongly influenced by the suitability of weather conditions for fluke transmissions in the key period over the summer (May to September). The nationwide prevalence of liver fluke is continuously changing and consequently the project is basing estimates of fluke prevalence in selected high prevalence flocks at a more conservative 50% as using a less conservative estimate risks not returning enough fluke infected sheep from a smaller total number of sheep to be sampled.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have pilot data available for the performance of the two initial diagnostic tests we will assess. This includes data from abattoir samples processed for the new Faecal Egg Counting based diagnostic test and pilot data from another project undertaken in conjunction with external collaborators in the case of the FlukEVal sera-based ELISA. These provide initial estimates of diagnostic performance for the tests which allow us to estimate the number of animals needed more accurately.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

By using sheep from commercial farm’s we reduce the need for transport and farm staff, with whom the animals may have a degree of familiarity, will be able to undertake much of the preparatory work with the animals including initial inspections of the health and welfare of animals before they are used as part of the project. The procedures have also been designed to minimise the amount of time that the regulated procedures will take to complete which includes minimising the time they are held alone or in small numbers and to reduce the time they are physically restrained by the researchers. This will allow us to release the animals into the final holding pen as soon as possible. Our protocol also

ensures that all the animals will be inspected by an independent veterinary surgeon shortly after completion of regulated procedures (within the same working day) where any delayed instances of adverse effects can be identified and any necessary action can swiftly be taken. The use of naturally infected animals negates the need to purposely infect animals in order to meet our objectives. As liver fluke is a parasite that can lead to serious health and welfare, purposely infecting animals does pose long term health risks if treatments are not swift and efficacious. In addition, the feedback on infection status from our regulated procedures will allow the farmers to administer appropriate treatments where necessary, bringing potential health and welfare benefits.

Why can’t you use animals that are less sentient?

We cannot use less sentient animals as the aim of the project is to evaluate diagnostic tests for liver fluke and rumen fluke specifically in sheep.

We cannot use more immature life stages as we require sheep with natural infections of liver fluke and rumen fluke and this only occurs in sheep that graze herbage (juvenile and adult sheep).

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

The procedures within this project closely resemble standard veterinary procedures that farmers and veterinary surgeons may use to diagnose live fluke infections in commercial livestock settings. We do not consider it appropriate to use refinements such as pain management as administering intramuscular anaesthetics will likely lead to harms equal to, or greater than, the mild procedures to be used in the project. Animals will be monitored for a period of time which can be extended at the discretion of the veterinary surgeon inspecting the animals after the end of the regulated procedures. However, harms to the animals are expected to be mild and transient.

It should also be noted that at least some of these animals will have naturally occurring liver fluke infections and there are potential welfare gains for these animals through correct diagnosis that farmers can use for treatments and therapies where appropriate.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The study will be conducted in compliance with the Committee for Medicinal Products for Veterinary Use (CVMP) VICH GL9 “Guideline on Good Clinical Practices”.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

The project leader will obtain information from qualified and experienced people at the university to obtain the lastest on 3Rs relevant to sheep. In addition, the project leader reviews the NC3Rs website regularly.

198. Analysis of thymus development and function

Project duration

5 years 0 months

Project purpose

- Basic research

Key words

Immunology, Thymus, Development, T cells, Tolerance

Animal types	Life stages
Mice	neonate, juvenile, adult, pregnant, embryo, aged

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

The aim of the project is to understand the genetic and epigenetic control of the development and function of the thymus throughout life. The thymus is the primary lymphoid organ where T cells are formed and selected.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Different congenital and acquired diseases that affect the thymus result in a loss of regular thymus function or compromise the generation of normal T cells. Insight gained from our studies will provide better diagnostic tools and rational approaches to the treatment of these pathologies.

What outputs do you think you will see at the end of this project?

The proposed work will provide several important outputs: New basic science information will be gained that will inform novel diagnostic approaches and form the basis for new therapeutic strategies to cure thymic diseases. The progress of this work will be regularly

published and presented at conferences, making its conclusions widely accessible to the wider scientific and medical community.

Who or what will benefit from these outputs, and how?

The translation of the proposed work will have several benefits. For developmental biologists and immunologists, the proposed efforts will provide novel information about which genetic and epigenetic controls are essential to form the thymus, an essential primary lymphoid organ. This information will be regularly made available throughout the course of the proposed project. For physician-scientists and clinicians, the generated information will help to decipher the so far unknown molecular causes of several thymus-related pathologies and thus provide the basis for targeted diagnosis and then a rationale for specific treatments. We anticipate that during the time of the project some of the information gained will be translated into direct clinical application. In the long-term, the project will provide invaluable basis for benefits to patients with thymus pathologies as the molecular origin of their diseases can be precisely defined and a rational therapy can be designed.

How will you look to maximise the outputs of this work?

The work is typically carried out as multi-disciplinary effort in collaboration with other scientists. The results are typically and regularly disseminated locally, nationally and internationally by way of conference presentations and publications. Interactions with colleagues that are part of the research network or attend conferences where the data is presented will provide insight into reasons for unexpected findings or unsuccessful approaches. Experimental findings will be shared with clinicians so that specific genes identified to play a role in development or function of the thymus can be investigated in patients to better define their thymus pathology.

Species and numbers of animals expected to be used

- Mice: 28,700

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

The project will use mice as these are the best studied animals for thymus research. To understand the genetic and epigenetic control of thymus formation we will study mouse embryos from day 10 of gestation onwards and newborn mice as thymus formation is only completed during the few days after birth. To judge thymus tissue maintenance and function, which is essential for the life-long formation and selection of T cells, we will analyse mice at different stages of their life trajectory.

Typically, what will be done to an animal used in your project?

The majority of mice will be analysed without having undergone any additional procedures, they will be humanely killed and analysis will be performed on tissue samples.

Irradiation or treatment with antibodies will be used to deplete the immune system in mice, they will then be given cells from the liver or bone marrow of donor mice, which will develop to restore the immune system. Some non-genetically altered mice will have undergone removal of thymus tissue at the breeder/supplier prior to be being supplied to the project. The mice that have undergone surigal removal of thymus tissue and mice with an inborn lack of thymus devlopment or function may have thymus tissues surgically grafted under the covering of the kidney. Animals will be immunised to study their immune responses to known antigens. Animals will be bred with gene mutations that will help us understand the development and function of the thymus. Blood and tissue samples will be taken to probe the cells of the immune system. Substances that induce an immune response in the body (antigens) will be givenby injection to probe the immune system's capacity to respond to antigens. All of the above could, but are not expected to, lead to infections, may transiently be related with pain, and will be associated for a short period with reduced well being as part of the recovery following surgery. Good surgical technique, pain relief, good husbandry, early intervention and treatment will be provided. All animals will be killed at the end of the experiment or as soon as an animal has been judged to have reached a humane end point, so that suffering is reduced. The expected level of severity for the protocols will be mild to moderate.

What are the expected impacts and/or adverse effects for the animals during your project?

Gene-edited mice may be born with a small or no thymus and may therefore experience a higher susceptibility to infections and/or autoimmunity. The former is unlikely to occur under specific pathogen-free conditions while the latter may be accompanied with some weight loss and/or organspecific impairment of functions due to autoimmunity that are typically mild or may not be clinically relevant at all. The injections will result in short term distress of the animal due to handling and will cause minimal pain owing to the needle stick. The surgical procedures will be carried out under anaesthesia but likely cause post-operative pain and discomfort which will be monitored and appropriate analgesia will be administered. Weight loss may occur after irradiation or treatment with an agent, Tamoxifen, which controls the expression of genes, but animals will given moist palatable food to ameliorate weight loss, if no sign of improvement (maintenance or gain weight) is seen within 48 hours the mice will be killed. Mice losing 15% of weight will be humanely killed immediately.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Of the 24,000 mice in Protocol 1, a mild breeding protocol, approximately 95%, will experience a subthreshold severity with <5% experiencing a mild severity.
Of the 4,000 mice in Protocol 2, a moderate breeding protocol, approximately 75% will experience a sub-threshold or mild severity, with potentially up to 25% experiencing a moderate severity.

100% of the 350 mice in the moderate experimental Protocol 3 will experience a moderate severity.

100% of the 150 mice in the moderate experimental Protocol 4 will experience a moderate severity.

100% of the 200 mice in the moderate experimental Protocol 5 will experience a moderate severity, as all will have undergone surgical removal of thymus tissue at the breeder/supplier prior to be being supplied to the project.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Thymus formation and maintenance are the result of a to date not fully understood complex process that involves many different cell types and their multifaceted interactions. This intricate development cannot be recapitulated in the test tube and thus necessitates studies in mice.

Which non-animal alternatives did you consider for use in this project?

While very few and limited aspects of thymus biology can be modelled in culture flasks using cell lines, the vast majority of biological questions in this project can only be informatively investigated in animals because non-animal alternatives do not exist as model systems to study thymus development, function and maintenance.

Why were they not suitable?

Non-animal alternatives have not been developed for thymus development and very likely will never be generated given the intricacy of the biological system that depends on multiple cell types to interact correctly with each other. Hence model systems do not exist to study a majority of questions related to thymus development, function and maintenance.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

We have carefully designed the experiments to be performed and subsequently calculated how many animals will be needed to realise in an informative fashion those experiments. Using breeding calculations suggested by the local animal work facility, we then calculated how many animals we need (i) to breed to obtain the numbers of animals required for the experiments planned and (ii) to maintain an animal colony over time.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible we will use littermates of appropriate genotype as controls. Based on past experience we will use the smallest possible group sizes that are still informative for thorough statistical analysis. As the effect of specific gain- or loss-of-function gene mutations and cellular manipulations are difficult to predict, power calculations cannot confidently be made ahead of time until results (e.g. phenotype, functions) are available from a small experiment, which will usually study 3-5 mice in the experimental (e.g. gene targeted) and 3-5 mice in the control group (wild type mice). Based on the findings of such studies, power calculations can be made for which advice from a biostatistician will be sought. Given the nature of our experiments, blinding and randomisation are in most cases not part of the experimental design and are therefore not planned.

If the phenotype caused by the mutation is unknown or cannot be predicted with certainty, then more animals will need to be investigated to establish the presence or absence of biological changes resulting from the mutation. But again, all possible efforts are undertaken to minimise the size of the experiment and hence the colony to firmly establish the pathomechanisms operational in these phenotypes.

Where different mouse strains may be required in parallel; strains may be crossed to make double or triple mutants, which will then be analysed in detail for molecular, cellular and organismic changes. We may breed mutants of the strains indicated with two genes mutated, when the single mutant phenotype is mild and in instances where the genetic epistasis needs to be established. In addition, we intend to visualise cells in selected tissues in which specific, targeted genetic changes have been induced using the expression and subsequent detection of distinct fluorescent proteins. Tissue targeting will be achieved by using the recombination of conditional alleles using Cre recombinase. We will set up a small number of breeding colonies to achieve the correct genotypes before expanding breeding for experimental use.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We will use multiple tissue from single mice which will allow not only the analysis of the cells and organs needed for a particular study but will also provide tissue that may be required for other downstream analyses or, for example, the establishment of new methods. These efforts are actively pursued to reduce the number of animals bred for tissue collection. Surplus animals and tissue will be shared with other groups should age, gender and genotype be appropriate. Notifications and requests are sent out by the animal facility via email to other laboratories in the local scientific community.

Our experiments are typically performed on a C57BL/6 genetic background which produces a comparatively large litter size. Consequently, fewer breeding pairs need to be used to have sufficient animals for the planned experiments.

Power calculations cannot confidently be made ahead of time until results are available from a small experiment, which will usually start with a pilot study 3-5 mice in the experimental (e.g. gene targeted) and 3-5 mice in the control group (wild type mice). Based on the findings of such studies, power calculations can then be made, using the Experimental Design Assistant tool or consulting a statistician.

Our molecular studies frequently employ new sensitive methods that require smaller cell numbers to achieve results or are based on single cell analyses that typically require fewer mice to be studied.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Wild type or gene-targeted mice on defined genetic backgrounds (inbred strains) will be used. A small thymus or its complete absence as a consequence of gene targeting does not impact the wellbeing of animals if kept under special pathogen-free conditions. The occurrence of autoimmunity as a consequence of abnormal thymus function is characteristically very mild in the mouse strains used for this project (C57BL/6) and is not expected to result in pain, suffering, distress or harm.

The majority of animals bred on this license will not undergo any additional protocols, but will be humanely killed and tissues will be examined in the laboratory.

Why can't you use animals that are less sentient?

Mice are used for the project as they constitute the genetically best studied experimental animal model for which relevant physiological and pathological data is already available. Moreover, reagents and methods necessary to investigate thymus biology are either only available in mice or best investigated in these animals.
Thymus formation in the mouse is initiated at day 10 of gestation and is only completed a few days after birth. Thereafter, different physiological changes (e.g., puberty, old age) influence the maintenance and function of the thymus tissue. As a consequence, mice at all ages during the life trajectory will need to be studied.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Specific aspects of the project will be continuously reviewed to achieve improved surgical techniques, to adopt alternative approaches, to shorten procedure lengths, and to improve pain management and post operative care (rehydration and feeding).
We will source mice that have had thymus tissue surgically removed (thymectomised) from a commercial company (e.g., Charles River) which performs this type of surgery far more frequently than we would and therefore have greater expertise. This should minimise the harms the animals experience.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

The practice will be informed by (i) published procedures of approved protocols; (ii) discussions with colleagues and experts in the field, (iii) newsletters by 3R organisations published in English, German and French

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

I subscribe to a 3R newsletters (https://www.nc3rs.org.uk/newsletter-archive and https://www.swiss3rcc.org/en/news-media/newsletters-archive), attend meetings where 3R advances are discussed and consult veterinarians and colleagues regarding new developments in replacement, reduction, and refinement as it pertains to my project.

199. Neuronal mechanisms of appetitive learning

Project duration

5 years 0 months

Project purpose

- Basic research
- Translational or applied research with one of the following aims:
    - Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
    - Assessment, detection, regulation or modification of physiological conditions in man,animals or plants

Key words

appetitive learning and memory, motivation and reward, associative learning and memory, neuronal mechanisms, addiction

Animal types	Life stages
Mice	adult, juvenile, neonate, pregnant
Rats	juvenile, adult, pregnant, neonate

Retrospective assessment

The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Exposure to signals or ‘cues’ (e.g. sound of ice cream truck) that are linked to rewards including palatable foods (e.g. ice cream) shape our urge and motivation to eat. For example, when we see a fast-food sign, we might be reminded about snacks and experience food cravings. Conversely, we also learn to suppress our responses to such cues when we update these linked cue and reward or ‘appetitive associations’. For instance, if that fast-food shop were to close down and no longer served food, we would react less to that fast-food sign the next time we see it. We do not fully understand how brain cells actually store, retrieve, and update these types of associations. The aim of this project is to investigate in rodents how a tiny minority of brain cells called ‘neuronal ensembles’ stores and retrieves appetitive memories, such as memories about food. We will study the cells in brain regions that are important for motivation to satisfy our basic needs (e.g. drinking and eating). We will also study at a molecular and cellular level, what is special about the brain cells that enables these appetitive memories to be formed, retained, and modified.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

On a fundamental level, revealing how brain cells store, retrieve, and update appetitive memories about is important for understanding basic survival behaviours, such as finding food. Additionally, this work has health-related implications, since desires such as food cravings that promote excessive eating are provoked by cues linked to food and food memory retrieval. Also, therapies that update associations about food and cues show some promise to suppress food cravings. By learning more about the cellular and biochemical properties of brain cells that store and retrieve appetitive memories and control appetitive states, we can better understand why certain individuals may engage in more excessive eating and gain weight compared to others. Also, indirectly and in the long-term (e.g. 15 years), these findings may help create better medicines for people that suffer from conditions such as excessive appetite, overeating, and obesity. This research is timely since, alarmingly, 2 out of 3 UK individuals are now overweight or obese, and are at higher risk of other diseases such as diabetes and cancer.

Moreover, since we are studying learned associations, this research will also provide clues on normal and abnormal mental functioning such as how the brain links and remembers other types of important information that shape our behaviours (e.g. the smell of smoke signalling fire). Such clues may also reveal more about conditions that affect our health such as drug abuse, since the development of drug addiction involves linking information about drugs and the environment where drugs are used (e.g. the sight of smokers creating a cigarette craving).

What outputs do you think you will see at the end of this project?

We envision outputs such as publications in open-access, scientific journals that reach a wide academic audience. Also, during the course of the project, we will present our work at both national and international academic conferences. Furthermore, we plan to reach out to the general public through the lab website, social media, and other local outreach events. Doing so will help disseminate our findings to a much wider audience, and enthuse their interest in learning more about the brain mechanisms of food memory storage and retrieval. Finally, any tools/approaches that are generated from this project will be made freely available to other researchers.

Who or what will benefit from these outputs, and how?

In the short-term (1-5+ years), the beneficiaries will be primarily academic. Since we are investigating mechanisms for appetitive learned associations and related behavioural states, there will be a wide variety of researchers who would benefit from our findings. These researchers may include experimental psychologists who are trying to determine the precise neuronal substrates of memories and aberrant behaviours and neuroscientists who are determining the molecular and physiological factors that control motivational states and shape and store memories. These researchers can use our data to further dissect the neuronal circuits that process memories and advance their research programmes. For instance, they may further characterise the molecular (e.g. neuronal surface receptors, neurotransmitters) alterations and neuronal activity patterns that occur

in response to storing and retrieving memories linked to rewards and any relevant changes in motivational states modulated by internal cues linked with these memories.
As an indirect, long-term (many years) consequence of this proposed research, whether the rewards being considered are food or drugs, this improved knowledge about the underlying neurobiology of appetitive associative learning and motivated behaviours could be used as a building block for further biomedical research that would impact the general public (both clinical and non-clinical populations) in several ways. First, clinical researchers can utilise this data to examine whether the molecular alterations that we observe in the rodent brain following appetitive conditioning are also present in the human brain (e.g. via post-mortem analysis or via PET studies). Subsequently, they can examine whether these factors are implicated in eating disorders, such as overeating and obesity. Second, by showing how learned associations about certain palatable foods (e.g. sugar) and their cues can alter certain rodent neurons, we may better educate people about what foods they should or should not eat. Such information can be valuable for researchers who wish to shape some aspects of public health policy. Third, we will be able to design more efficacious therapeutics that stimulate or inhibit specific neuronal populations that encode appetitive memories and motivation. As such, researchers in drug discovery may benefit from our research. Using such treatments, we may stimulate appetite in those suffering from disorders such as cancer, or decrease appetite for those suffering from overeating.
To provide some perspective about the widespread nature of eating-related disorders, the number of people suffering from eating disorders in the UK alone is estimated to be over 10 million. These disorders are also associated with other illnesses that such as diabetes and heart disease. Also, since our research has implications for other conditions such as age-related memory decline and addiction, those researchers in these areas will benefit as well.

How will you look to maximise the outputs of this work?

We will maximise the outputs of this work by finding new local and/or international collaborators who will be able to expand our research programme into a more innovative direction by offering their conceptual and practical expertise. To illustrate an example, our recent work was greatly enriched by establishing a collaboration with a local researcher at our university who provided his expertise on live brain imaging, which revealed novel brain activity patterns that helped establish food memories. Also, such innovative collaborations open new doors to apply for funding schemes that allow for more impactful and ambitious research to be conducted.

Furthermore, we will disseminate our work in open-access journals that have a wide reach and make an active effort to promote our work through social media platforms and blogs. We will also ensure that our research is highly accessible by producing a graphical abstract of our key experiments and findings. Wherever possible, if a story is newsworthy, we will contact the media team out our university to further promote our studies. Finally, we will aim to publish any negative findings from experiments. Doing so will help prevent other scientists considering the same research question from performing those negative finding experiments, preventing unnecessary animal usage (Reduction) as well as time and financial resources.

Species and numbers of animals expected to be used

- Mice: 9000
- Rats: 1900

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

We will use primarily use adult male and female mice and rats to examine how appetitive memory mechanisms operate in the brain. We have routinely used these animals previously, which are readily trained and perform robustly on the behavioural tasks described in this application, reducing the number of animals used in line with the 3Rs. Rats and mice are an appropriate choice of species because their neural circuits that process information related to food and their associated cues as well as motivated behaviours to seek food are remarkably similar to those of humans. Hence, findings from these studies which reveal novel cellular and biochemical profiles of these neural circuits have translational applicability. In some cases, rats are preferred to mice, since their brains are bigger and as such specific brain areas are easier to manipulate with intracranial procedures. In many cases, we will use mice since many genetically modified mice are available that allow activated neurons to be further characterised and manipulated. For instance, by using such mice we can monitor the activity of fluorescing neurons in a live broadcast manner and/or characterise the electrical properties of activated neurons. We will use adult mice to better compare the findings obtained from our studies with our previous finding as well as in the literature. However, in some cases we may wish to perform similar studies in adolescent mice to determine whether their brains process food memories differently or similarly to adults.

Typically, what will be done to an animal used in your project?

Our project is divided into 3 main workstreams. In the first two workstreams, animals will typically undergo an appetitive learning procedure under freely moving conditions that will last a few weeks where they have to learn 1) that a cue such as an auditory signal would predict food availability; or 2) to perform an action, such as a lever press, to obtain food. We will probe for their food memories by measuring the responses to the cue in the form of food seeking or by measuring lever presses in the absence of food availability.
During this learning task, animals undergo mild food restriction, so that they become motivated to learn this task. In the first workstream, animals 9-13 weeks old will undergo the appetitive learning procedure. Then at various time points during the learning procedure, we will euthanise the animals and then examine their brains to measure the cellular and biochemical properties of neurons that were activated during learning. In addition, some animals will undergo brain surgery prior to the appetitive learning procedure (or naive state) such that specific neurons can be further characterised and/or controlled using genetic transformation or other biochemical methods. Some animals will simply undergo mild food restriction without undergoing any appetitive learning procedures, in order to examine how the brain encodes an appetitive state (e.g. hunger state). Doing so will help us understand how the brain processes internal hunger cues, that influence the formation of learned associations.

In the second workstream, animals 9-13 weeks old will undergo brain surgery such that the live activity patterns of brain cells can be examined using optic fibres or a cranial window. Following recovery from surgery, they will undergo the above appetitive learning procedure. For some animals, several times during training or in the memory test, we will connect optic fibres to the cannula. Then we will measure activity patterns from labelled

neurons to examine what brain activity may help memory retrieval and/or manipulate the activity of these neurons using optical approaches to see if these neurons are involved in the actual memory retrieval. Some animals will undergo implantation of a cranial window onto its skull. After recovery from surgery, animals will be gradually habituated to being held in position under a microscope on top of a treadmill or cylinder, allowing the mouse to walk or run freely. Over a course of 1-2 months, we will perform around 10 imaging sessions to monitor brain activity, separated usually 2 days apart at various time points in the appetitive learning procedure.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals will undergo mild food restriction and will lose a slight amount of weight (10%) for several weeks to motivate them to learn the behavioural task. Their weights and body condition will be monitored daily. However, this weight loss has actually shown to improve the lifespan and health of animals. In the event that there is more weight loss than 15% of their normal weight, we will immediately limit or suspend the food restriction to avoid any suffering.

Animals that undergo surgery might experience pain afterwards. However, these can be managed well with pain medication that humans also respond well to. Initially following surgery, animals are subdued, but they rapidly recover over a period of several days. If necessary, during the recovery period, we can give them soft food to aid their digestion and more pain medication. The health of the animals will be monitored, and if their health deteriorates, then they will be euthanised within 3 days or in severe cases, immediately.

Animals will also receive injections of various substances such as those that turn on certain genes in the brain and/or to control appetite. Discomfort is present during these injections, but only last less than a minute and we use the finest needles possible that minimise pain.

Animals may be initially stressed when connecting an optic fibre cable to the cannula or getting used to being held in a head restraint under the microscope. However, they are slowly accustomed to this process over several days in a gradual manner, resulting in very brief (1 min) periods of stress.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

Mice
Total proposed number in project: 9,000
Proportion of severity: 53% mild, 47% moderate

Rats
Total proposed number in project: 1,900
Proportion of severity: 53% mild, 47% moderate

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Both rats and mice are ideal model organisms for these types of studies as they have similar brain circuits that generate memories and create motivated actions similar to humans. Our work requires rats and mice because we wish to examine the relationships between the activity patterns and cellular and biochemical profiles of specific groups of brain cells that are implicated in food memories. This requires studies in the intact brain in a behaving organism. However, such experiments cannot be performed in humans due to ethical and methodological considerations. For instance, brain imaging techniques such as fMRI lack the resolution to visualise the activity of specific groups of neurons. Moreover, these studies cannot be performed using cell culture systems because they do not allow us to examine properties of brain cells that are directly relevant to memory retrieval and the diversity of brain cell types is not represented in cell cultures. Also, computer simulations can mimic some aspects of learning, but they will not reveal the unique cellular and biochemical properties implicated in food memories.

Which non-animal alternatives did you consider for use in this project?

We have considered cell culture systems and computer simulations. However, as mentioned above, these systems do not allow for the direct examination of activity patterns and cellular and biochemical profiles of neurons that are relevant for food memory retrieval. As such, they cannot inform us of the properties of specific groups of neurons implicated in behaviours and responses that depend on food memory retrieval such as seeking out food and food cravings.

Why were they not suitable?

The mammalian brain is composed of millions (to billions) of neurons that undergo cellular and biochemical changes during learning and this complex native circuitry cannot be completely mimicked in cell culture systems. Also, unlike the brain, the diversity of brain cell types is missing in cell culture, and for instance we cannot specially examine if excitatory neurons specifically interpret information about food-linked stimuli. Computer simulations could not be used here since they do not reveal the physiological properties of specific neurons implicated in food memories. Additionally, there are no computer simulations to date that completely mimic the reward and motivation brain circuits in an animal. To build better computer simulations, data from animal experiments are necessary. Finally, unlike an animal both cell cultures and computer simulations are not capable of learning the relationships between actual sensory stimuli and food rewards.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise

numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

The numbers are based on how many mice were used in my last license. We are providing an overestimate here to account for expansion of our any new collaborative research programmes that may form during the course of the project. If necessary, we will adjust the number of animals on the license with amendments if our estimates need further calibration. Our typical sample size per group ranges from n=7-12.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

Wherever possible, we use a within-subjects experimental design to collect multiple data points and to increase statistical power and reduce the usage of animals. Such data can be collected for behavioural parameters such as food seeking as well as neuronal activity data.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

We have optimised our breeding strategy such that the minimal number of mice are necessary for efficient generation of transgenic animals. Moreover, the non-transgenic, wild-type littermates will be used for pilot studies or actual experiments since they are similar to normal animals by our laboratory and/or by other laboratories. As mentioned above, wherever possible we will also collect multiple data points from a single animal at the behavioural and neuronal levels to increase data yield. Before a new study is conducted, we will conduct a thorough literature search and use our past data to enable us to conduct pilot experiments with the most optimal parameters for measuring reliable behavioural and neuronal responses. Based on the observed effect sizes, we will determine whether an experiment can be completed with a reasonable sample size. If this is not the case, we will not proceed with that experiment.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

In addition, to normal ‘wild-type’ rats and mice, we will use transgenic rats and mice that express special proteins that allow for further characterisation and manipulation of activated brain cells. Although these animals are genetically altered, there is no harmful phenotype and published studies from our lab and others demonstrate that they also behave similarly to normal mice and rats.

This project involves complex behavioural tasks (e.g. learning to make a particular response such as pressing a lever to obtain food) and rodents perform these tasks very accurately. During behavioural training, we carefully observe the animals using a live camera feed, and by examining their training data. These observations are useful for detecting if animals might be unwell. In addition, many aspects of the rodent brain are similar to humans, including those brain areas are important in producing the behaviours that we study. We reduce stress to these animals through use of food rewards in training, rather than punishment. Training using food rewards can be very successful and is widely used to train service animals (e.g. police dogs).

To improve their well-being, animals will be group housed most of the time as rats and mice are social species which naturally choose to live in groups. We also house these animals with enrichment in their home cages and handle them frequently so that they are used to the experimental processes. Also, they will usually undergo behavioural tests in the same familiar location in a dark and quiet room that minimizes stress.

Many of our experiments will involve performing surgery to insert a fine tube called a cannula through the skull or insert a small window in the skull. Animals usually recover well following these procedures, and exhibit behaviours such as grooming, eating, and drinking many minutes after waking up from the anaesthesia. In general, we pay lots of attention to the well-being of the animals (especially after surgeries), including trained and qualified personnel examining a wide variety of behavioural and physical signs. If necessary, animals that are unwell would be humanely euthanised to prevent excessive suffering. These animals are handled frequently so that they are not startled by human contact, and that this also acts as a form of enrichment. Animals are also habituated or get used to the brain activity recording apparatus in a gentle and gradual manner over days, to minimise any stress.

By doing so, any brain activity data obtained is more reliable.

Why can't you use animals that are less sentient?

We need to use mice and rats here as these animals possess brain areas with brain cells that process information related to motivation and reward similar to humans. These animals are necessary to study how brain cells in these brain structures communicate with each other when storing and retrieving food memories. Less sentient, invertebrate organisms such as snails are capable of storing memories about food similar to mice and rats. However, as they are not mammals their brain cells and circuits are rather different to that of humans and lack the richness and complexity. Hence, compared to studies on rats and mice, findings from these studies are not as directly relevant for understanding the human condition for phenomenon such as appetitite control, food cravings, and excessive eating. Moreover, brain cells from lower beings such as invertebrates contain receptors that may not be on brain cells from mammals. As such, they are not informative for developing better therapeutics that potentially modulate human brain function.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

As in our previous studies, wherever possible, we will continue to refine our methods to improve the well-being of animals, as soon as we learn of more optimal approaches. For instance, for our behavioural experiments, we may measure additional behavioural parameters that indicate that the animals are learning the task optimally, which will help

guide when to best take the brain activity measurements. Doing so will minimise unnecessary stress that may occur from live brain imaging procedures. Additionally, we will increase habituation to the testing context to decrease the incidences of any non-specific behaviours in order to increase the signal-to-noise ratio of our behavioural parameters of interest. We may also adopt new methods which will help better stabilise our cranial attachments, so they adhere better to the skull. For instance, this year we sought advice from a research group in UCL and managed to obtain a more stable adhesive for cranial attachments which has greatly reduced the chances of these coming off. Also, in the previous years we sought advice from more experienced people regarding how to score the skull better to improve this adherence. Finally, all mice will be removed from cages using tunnel handling to minimise stress of tail handling.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will continue to regularly update our knowledge on refinement using peer-reviewed, academic papers that use similar experimental methods and mouse lines. We will also refer to resources, such as webinars and publications from organisations such as LASA and NC3Rs, including the LASA report on avoiding animal mortality, LASA guide to aseptic techniques, and NC3Rs newsletters. Moreover, we regularly subscribe to LabAnimal magazine that will contain relevant articles related to refinement. We will also consult with mousewelfareterms.org so that we can better learn about the terms that describe mouse health for improved assessment and more precise communication of mouse welfare.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

As someone who used to teach the ethics of animal research, I am interested in following updates from:

The Universities Federation for Animal Welfare (UFAW), The Laboratory Animal Science Association (LASA) and the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). We regularly receive updates from these organisations. These organisations often provide educational material on how to improve animal well-being and minimise harm, and currently I encourage my laboratory members to attend webinars hosted by NC3Rs. I will also have regular meetings with my laboratory members to critically examine if any of these good practices can be adapted without altering our experimental outcomes. For instance, in the past we have added additional enrichment into the animals' home cage and determine if this would affect food seeking behaviour. Since a pilot study determined that it did not, we have adopted this refinement. Additionally, my laboratory members regularly search the literature and/or seek help from others to improve experimental protocols to improve data yield. Doing so contributes to the reduction in animal usage.