Home Office

NON-TECHNICAL SUMMARY

Using zebrafish to understand neurodegenerative disease and develop therapies

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) 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

zebrafish, development, disability, therapy, mechanism

Animal types	Life stages
Zebra fish	embryo, 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 is the aim of this project?

This project aims to increase our understanding of diseases and the functions of the mutated proteins that cause those diseases, as well as providing disease models for therapeutic discovery and 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?

The project focuses on rare inherited diseases, which together affect about one in every 10 people, even though each disease has an incidence of less than 1 in 2000 people. They frequently cause premature death in childhood and the vast majority have no approved treatments. We will also study diseases that have a more complex origin, in which the same genetic factors interact with environmental factors. There is a huge need to find treatments for both inherited and complex diseases.

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

We expect to have increased our understanding of the function of three or more proteins involved in childhood neurodegeneration and epilepsy, including understanding how those proteins interact with other processes within the cell and how disease arises. We plan to generate several disease models, and by studying them we will better understand the disease process. One or more chemicals will be tested to see if they treat any of the childhood neurodegenerative diseases that we are studying. We will publish our findings. We will apply for orphan drug designation for a drug to treat seizures in a childhood neurodegenerative disease called CLN2 disease. We may apply for other orphan drug designations or patents for potential drugs.

What will be the impact of this proposed work on humans / animals / the environment in the short-term (within the duration of the project), in the medium-term and the long-term (which may accrue after the project is finished)?

In the short-term scientists will benefit from increased understanding of childhood neurodegenerative diseases including the normal function of the protein that is mutated in the disease, disease pathology and the effect of the disease on processes occurring within cells. This will lead to better treatments for patients in the long term.

The availability of additional disease models will enable treatments to be tested on more models so that the research community can be more confident that the treatment will work on patients, who will benefit in the medium term.

The availability of embryo-larval zebrafish disease models that can be used to test thousands of chemicals in a relatively short amount of time means they can be exploited to find novel chemicals with potential to treat the disease. This will benefit scientists in the short-term and patients in the long-term.

The knowledge of the mechanism of action of the hit compound that appears to treat seizures in CLN2 disease, will support efforts to bring the compound to the clinic in the medium term, and provide researchers with novel disease and treatment mechanisms in the short-term.

How will you maximise the outputs of your work?

We will publish our work as open access. For standalone drug testing we will publish Registered Reports, provided we have our collaborators consent. For other standalone drug testing, we will publish negative results. We will disseminate our research at international conferences.

We collaborate with others using different models or methods to extend the impact of, and increase the confidence in, our research.

Species and numbers of animals expected to be used

- Zebra fish: 21400

Predicted harms

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

Describe, in general terms, the procedures animals will undergo, eg injections, surgical procedures. Include the typical number of procedures individual animals will undergo and the likely duration of suffering.

We will study existing and new disease models, which we will create. To create new models, we inject the one cell embryo with agents to specifically alter a targeted gene. We select healthy embryos and grow these to adulthood. We then select adult fish carrying this genetic alteration in their sperm and eggs, and breed from those fish to generate a new generation (as many generations as is needed). During these breeding programs, we need to be able to identify fish carrying the genetic alteration from wildtype fish, so we take a small number of cells (either from their fin which regenerates, or from their mucus on their skin) and analyse the gene for the alteration. Once we have identified the genetically altered fish, we incross them to make fish that have two genetically-altered copies of the gene, which we expect to show signs of the disease at some point during their life. We then assess those potential disease models to see if they do actually show signs of the disease, and if so, we study them further, and set a humane endpoint.

To understand the disease mechanisms, we sometimes need to manipulate other processes as well, either using pharmacological agents or by altering other genes. We then examine the fish to see if this has made a difference. We can examine their movement or behaviour, or an invisible process happening within, which we visualise with a label (often a chemical or a fluorescent tag), most often on fish that have been sacrificed for their tissue.

To search for treatments, we use embryonic disease models to screen large numbers of chemicals, and check to see if the signs of the disease are reduced by adding the chemical to the water the fish are in. If we already have good reason to believe that a chemical might make a good treatment (eg data from cellular disease models), we might use adult disease models but we will use embryonic and larval models first if possible. In this case we might bathe the fish in the chemical, or inject it. If our results are similar to those of our collaborators that have models of the same disease in other species or in cells, we can be more confident that the treatment will be beneficial for patients, or that the disease mechanism is conserved and important.

As a practical way to reduce the numbers of fish kept alive, we take sperm from adult males and freeze it. We can then use this to re-derive the strain several years later using *in vitro* fertilisation (where eggs are taken from a female adult and mixed with the sperm to generate embryos that will grow into adult fish). This also reduces the effect of genetic drift on experimental results.

Expected impacts or adverse effects on the animals - for example, pain, weight loss, inactivity or lameness, stress, or abnormal behaviour - and how long those effects are expected to last.

Most adverse effects are expected to be transitory eg when taking mucus or fin samples there may be slight discomfort, ameliorated with analgesia. Sometimes we will need to anaesthetise the animals eg when taking mucus or fin samples. performing electroretinography or electroencephalography, or when imaging, and there is a small chance of an adverse reaction, in which case we will cull the fish straight away. It is possible that taking mucus or fin samples, massaging the belly to release eggs or sperm, or performing transplants could result in infection, in which case we will cull the fish as soon as this is noticed, or treat with antibiotics in the case of transplants. Massaging the belly to release eggs or sperm could also result in damage to internal organs if not gentle enough, in which case we will cull the fish as soon as this is noticed.

The disease models are likely to show one or more of the following clinical signs: obviously small eyes; obvious weight loss; discolouration; abnormal swimming, behaviour, posture or balance; abnormal respiratory pattern; unusual interactions such as aggression. We will cull all fish which have shown clinical signs for 4 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 species)?

About 50% of fish will experience mild severity and 50% will experience moderate severity.

What will happen to the animals at the end of the study?

- 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 model a disease, investigate the pathological steps, search for therapies and test those therapies, you need all the relevant tissues present. The diseases we work on predominantly affect the central nervous system but there is increasing evidence that they affect the peripheral nervous system, and heart, and we know that the mutated proteins function in all cells, so we expect pathology to arise in other tissues if we manage to treat the brain and improve survival. Furthermore, in some of these diseases, it is known that patients generate antibodies in the periphery that bind proteins in their brain. Hence, a full understanding or the disease and treatments will only come from studying intact animals.

What was your strategy for searching for non-animal alternatives?

We employ replacement strategies where possible. These include performing experiments in cell models (traditionally used mammalian cells, patient cells, human stem cells, the social amoeba), invertebrates such as the fruit fly and the nematode worm, frog oocytes and/or embryonic zebrafish depending on the availability of suitable models or whether the gene can be easily identified and mutated. We also take tissue from culled zebrafish and grow the cells in a dish.

Why were they not suitable?

These methods do not replicate the complex environment seen in a vertebrate organism or the situation at post-embryonic stages but they do give us some information so we do these experiments to inform what experiments should be performed in zebrafish at protected ages.

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 use up to 8000 of these fish for breeding purposes to supply embryos for experiments. This is based on 40 different strains (some are combinations of several strains, for which several intermediate strains need to be generated) with no harms caused by the genetic manipulation. Zebrafish are healthier when in groups of 20 or more, and new generations are needed about every 6 months - the old generations are not culled until the new generations are genotyped (usually as adults). For some complex strains, only a small proportion of the fish are the correct genotype but this is not known until adulthood when we can genotype them. Over a 5 year period, this adds up to 8000 fish.

We expect to use 8000 (over a 5 year period) post-embryonic genetically-altered strains for simple experiments where we document the harmful effects of the genetic alteration. Each experiment requires a pilot study to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 75 animals (25 animals in each of three groups) per replicate, this allows for about 35 experiments over 5 years.

We expect to use 4000 (over a 5 year period) post-embryonic genetically-altered strains for complex experiments where we document the potentially harmful effects of the genetic alteration in combination with a drug or chemical treatment, for example. Each experiment requires a pilot study to calculate the number of animals required for a full study, and then each study should be replicated three times to ensure reproducibility. Assuming 150 animals (25 animals in each of six groups) per replicate, this allows for about 9 experiments over 5 years.

We will need to generate new strains of zebrafish and estimate 1000 animals will be needed. This is assuming 5% of animals will be able to transmit the new genetic alteration to their progeny and that we may make up to 50 strains.

In order to preserve newly created strains, we expect to use 400 animals, which is approximately 10 animals for 40 new strains or combinations of strains.

What steps will you take to reduce animal numbers? Where applicable, what principles will you use to design experiments?

We have used the Experimental Design Assistant to design experiments using zebrafish embryos and will continue to use it for all zebrafish experiments. This has led to us reducing bias by introducing randomisation and blinding (where possible, given that some phenotypes are visible). For each experiment we also write 1) the objective, 2) a detailed description of the method and reagents needed (and ensure they are available) to minimise variance between researchers, 3) a calculation of the numbers of animals needed based on pilot studies, and 4) a description of the analysis method and the statistical test to be used.

We preserve sperm to reduce live animals, negating the need to recreate strains or combinations of strains, and reduce genetic drift. We use live animals for gamete production to reduce animal numbers.

We aim to trial genotyping embryos < 5dpf using the Zebrafish Embryonic Genotyper, which gently removes cells from the skin for genotyping. Fish are not harmed and can be raised to adulthood without having had an invasive genotyping method. Significantly, only fish or the required genotype need to be raised to adulthood, reducing numbers.

What other measures apart from good experimental design will you use to minimise numbers?

Pilot experiments will be used to generate data for a power calculation to determine a suitable number of animals for a full replicate. For experiments using embryos, we usually find that 10 per group is acceptable unless the experiment is reporting locomotion or behaviour, which appears to be much more variable and sometimes requires 30 animals. Simple experiments comparing wildtype to a disease model tend to require less animals than an experiment where there is also a drug treatment, where the drug treatment may not completely rescue the signs of disease, so in these cases, more animals are usually needed. For experiments new to us, we discuss animal numbers and data analysis with statisticians in advance.

We estimate the number of animals needed and breed what is required plus 20% to account for deaths during larval stages. Once zebrafish are genotyped, those not required for experiments or breeding are culled. We aim to genotype using the earliest, least harmful method possible.

We offer to share spare animals and tissue at our institution. They are often used for Schedule 1 training or as sentinels.

We do not keep animals beyond 18 months old and frequently cull them earlier if the next generation is genotyped and has reached adulthood.

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.

Why are the animals, models and methods you will use the best to meet your objectives? Why will your approach cause the least pain, suffering, distress or lasting harm?

We use zebrafish as there is now a great deal of evidence that data from this vertebrate can be extrapolated to humans due to the level of genetic conservation, conservation of relevant anatomy and highly similar responses to drugs. The models we have generated to date for CLN3 disease, CLN2 disease and East syndrome are no exception. In support of this conservation, we have evidence that a novel drug first tested in cells works similarly in mouse and zebrafish to reduce CLN3 disease symptoms. The fast, external development of the transparent embryo means that many disease models can have clinical signs during unregulated embryonic and larval stages, and these can be viewed easily without harming the mother. Where possible, unregulated embryonic and larval zebrafish will be used.

The CLN2 disease model will be used before the regulated age. The CLN3 disease mutant model causes only mild symptoms (thinner retina and yellow colour) in adults by the humane endpoint chosen. We do not yet know when the new disease models will display symptoms, but we aim to make models that replicate the genetic change in humans as best we can so that the model is as relevant to the disease as it can be.

The majority of animals will be maintained as non-harmful genetically altered families, minimising suffering. Animals that are maintained as harmful genetically altered families will predominantly be used for experiments that require us to observe the harmful consequences of the genetic alteration, though we aim to limit the harm caused as much as possible by choosing to study the earliest relevant consequences. Occasionally, we only have one genotyped animal for a particular strain. To avoid single housing, we will house them with animals of another genotype that have a visible difference such as fin length, pattern or coloration.

Some of the animals that will display harmful effects of the genetic manipulation will be further manipulated with genetic material, chemicals or drugs, or by transplantation of tissue. Such experiments are required to show a drug treats the disease, or that a disease process really has negative consequences and should be targeted in treatments, or which cells need to be corrected by gene therapy. Transplantations will be performed during unregulated embryonic or larval stages to minimise harm and because the tissue integrates better into the host at this stage. Drug, chemical or gene therapy treatments will be first tested on unregulated embryos and larvae to determine the effective dose and frequency where possible, if there is no relevant information in the literature, and to confirm finding in the literature. The least invasive methods of delivery will be used where possible (bathing, in the food, oral gavage, then injection), depending on what is known about the metabolism of the agent, where it needs to be delivered to, and how it might be delivered in humans. In these experiments we also aim to limit harm by choosing to measure the earliest harmful effects. We will create new disease models and genetically-altered strains. Only healthy fish are raised to the regulated age, to minimise harm when doing this.

Fish will be examined for the effects of their genotype or treatment. The majority of these investigations will be observations of normal behaviour and locomotion or tests on the tissue after euthanasia. We will perform some neurophysiological tests that require anaesthesia or restraint but are otherwise not harmful. The dose of anaesthesia is carefully titrated for the first fish (ensuring a quick and healthy recovery after the procedure) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

All these strains will need genotyping. We will use the least harmful method (fluorescent microscopy or deduction of genotypes from the offspring as observed through microscopy during unregulated stages) and the earliest stage possible. When tissue samples are required, we will preferentially use skin swabs over fin clippings. Both of these methods use anaesthesia with recovery, but skin swabs are thought to be less harmful. We have recently begun using skin swabs and will need to show that the results using this method is as good as with fin clips for each strain before we can use skin swabs exclusively. The dose of anaesthesia is carefully titrated for the first fish (ensuring a quick and healthy recovery) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish. Analgesia is used prior to, during and after fin clipping.

We will need to preserve sperm, which allows us to freeze these strains and maintain less fish alive. These strains can later be resurrected using in vitro fertilisation. Again, the dose of anaesthesia is carefully titrated when taking sperm or eggs. Fish recover and can be used to provide sperm or eggs once more, meaning less fish are required. Currently, experts at another university preserve sperm for us. We aim for them to train us in this method.

Why can’t you use a less sentient animal, (for example at an immature stage, a less sentient species or using terminally anaesthetised animals)?

Where possible, we will use animals at more immature life stages. This will depend on the stage when the genetic manipulation causes changes that are measurably different from wild type zebrafish. When we do not need to measure a clinical sign, we will measure changes that be observed in tissue from animals culled or terminally anaesthetised prior to the age when the clinical sign is evident. When we do need to measure a clinical sign, we will design experiments to assess as many changes as possible (both live and under terminal anaesthesia) to gain maximum data from each animal.

We favour the use of zebrafish over invertebrates as the zebrafish brain is considerably more similar to the mammalian brain. As zebrafish are the genetic vertebrate model with lowest neurophysiological sensitivity, they are also our preferred vertebrate for experiments at regulated stages. We feel that the zebrafish is the most refined model for the research questions we are addressing.

What are you going to do to refine the procedures (for example increased monitoring, post-operative care, pain management, training of animals) to minimise the welfare costs (harms) to the animals?

When studying adverse effects, we aim to limit the harm caused as much as possible by choosing to study the earliest relevant consequences.

Occasionally, we only have one genotyped animal for a particular strain. To avoid single housing, we will house them with an animals of another genotype that have a visible difference such as fin length, pattern or coloration.

Transplantations will be performed during unregulated embryonic or larval stages to minimise harm and because the tissue integrates better into the host at this stage.

Drug, chemical or gene therapy treatments will be first tested on unregulated embryos and larvae to determine the effective dose and frequency where possible, if there is no relevant information in the literature, and to confirm finding in the literature. The least invasive methods of delivery will be used where possible (bathing, in the food, oral gavage, then injection), depending on what is known about the metabolism of the agent, where it needs to be delivered to, and how it might be delivered in humans.

We will create new disease models and genetically-altered strains. In this method, only healthy fish are raised to the regulated age, to minimise harm.

Once we have created a new genetically-altered strain, we will develop a score sheet for that strain so expected phenotypes are known and specifically looked for.

The dose of anaesthesia is carefully titrated for the first fish (ensuring a quick and healthy recovery after the procedure) and then tested on the next two fish to confirm the dose is correct, before continuing with the remaining fish.

We will use the least harmful method (fluorescent microscopy or deduction of genotypes from the offspring as observed through microscopy during unregulated stages) and the earliest stage possible. When tissue samples are required, we will preferentially use skin swabs over fin clippings. Both of these methods use anaesthesia with recovery, but skin swabs are thought to be less harmful. We have recently begun using skin swabs and will need to show that the results using this method is as good as with fin clips for each strain before we can use skin swabs exclusively. We use analgesia when we take fin samples.

We aim to trial genotyping embryos < 5dpf using the Zebrafish Embryonic Genotyper, which gently removes cells from the skin for genotyping. Fish are not harmed and can be raised to adulthood without having had an invasive genotyping method.

The protocols state the minimum required monitoring provided. Monitoring will be increased if any fish respond differently than normal to the procedure.

What published best practice guidance will be followed to ensure experiments are conducted in most refined way?

We will follow Home Office guidance. For published methods, to ensure best practice and the most refined method is being followed before implementation, we will discuss new methods with the Named Animal Care and Welfare Officer and possibly the Named Veterinary Surgeon.

How will you ensure you continue to use the most refined methods during the lifetime of this project?

We seek advice from large zebrafish facilities and keep abreast of latest refinements via the British Association of Animal Husbandry, the European Zebrafish Meeting, the Lab Animal Science Association, the NC3Rs, the Institute for Animal Technology and the journal Zebrafish. We receive newsletters and bulletins from these groups. We search the literature for more refined methods. I am a committee member for EUFishBiomed, members of which have recently reviewed use of anaesthetic.

We have meetings every few months with other research groups using fish and the staff that care for the fish and present our research so they can feed back on any best practice they are aware of.

We will keep up to date with changes in the ARRIVE guidelines for the reporting of animal experimentation.

When we find a new method we believe to be best practice, we contact the developer and arrange for training.

Explain the choice of species and the related life stages

Data from zebrafish can be extrapolated to other vertebrates due to a high level of conservation, particularly biochemical, cellular and developmental. Responses to pharmacological agents is also similar. This means that it is valid to use the zebrafish to model disease, investigate disease mechanisms and search for and test novel treatments. The majority of the disease models we use are for inherited neurodegenerative diseases and epilepsy, so in this case we also know how conserved

the gene is from zebrafish to humans. The rapid, external development of zebrafish enables the vast majority of experiments to be performed at developmental stages which are not regulated by the Home Office without harming the mother. Thus we feel that the zebrafish is the most refined whole animal model for our research and that, of the available vertebrate models, it has the lowest brain function.