1.1 Information on the scientific background

1.1.1 Indication of the purpose of the experimental project and scientifically justified statement that it is to be assigned to one of the purposes mentioned in Section 7a, Paragraph 1 of the Animal Protection Act.

[…]

Explanations [~400 words]:

This project is aimed at characterizing the effects of microbiota transplantation on age-related neurodegeneration in the brains of killifish. The relevance of the project stems from the fact that this vertebrate species offers the unique opportunity to study, in a very short time for a vertebrate (i.e. 3 months), the effects of environmental manipulations that can affect neurodegeneration.

Recent findings have proven that the intestinal microbiota has a key role in modulating neurodegenerative phenotypes in mice models of neurodegeneration (Sampson et al., 2016). However, these mouse models rely on the transgenic induction of neurodegeneration, rather than resulting spontaneously as a consequence of ageing. Killifish have recently been shown to spontaneously neurodegenerate with ageing, as seen in human Alzheimer’s/Parkinson’s patients (Matsui et al., 2019). Therefore, using the short-lived, naturally neurodegenerating killifish model, we are in a unique position to ask whether altering the intestinal microbiota can affect the natural onset of neurodegeneration.

1.1.2 Scientifically justified presentation of the indispensability of the experimental project, taking into account the current state of scientific knowledge (Section 7a (2) No. 1 TierSchG in conjunction with Section 31 TierSchVersV))

- Please attach non-technical project summary (ID-No .:) (§ 31 Paragraph 2 TierSchVersV);

- not required for advertisements -

Explanations [1 page]:

Brief summary of the objectives of the experimental project [1/2 page]:

Gut dysbiosis, a condition in which microbial homeostasis is disturbed, has been suggested to causally affect Alzheimer’s disease (AD) and Parkinson’s disease (PD) (Kim et al., 2020; Sampson et al., 2016). Although effort is being made to understand how gut dysbiosis affects AD, the current animal models largely rely on trans-genic induction of Aβ42, limiting research into the causal effects of gut dysbiosis on the onset of Aβ42 aggregation and AD. Here, I propose to characterize the causal effects of gut dysbiosis on neurodegeneration using the African turquoise killifish (*Nothobranchius furzeri*). *N. furzeri* have been shown to undergo spontaneous neurodegeneration, including Aβ42 aggregate formation, within 4 months of life (Matsui et al., 2019). Interestingly, *N. furzeri* also develop age-related gut dysbiosis which greatly affects their lifespan, as microbiota transplantation from young to aged fish prolonged lifespan by 40% (Smith et al., 2017). Here, I will investigate whether microbiota transplantations can be used to delay or prevent neuro-degenerative phenotypes in *N. furzeri*, asking “What is the causal role of gut microbiota in the onset of neurodegeneration?”. The Valenzano research group at the Leibniz Institute on Aging – Fritz Lipmann Institute (FLI) in Jena currently uses *N. furzeri* as its main model organism and will be the place where all the proposed experiments will be performed.

Brief summary of the research objective for the proposed study:

I will use microbiota transplantation from young donor fish to aged *N. furzeri* fish (Aim 1), from neurodegenerative *N. furzeri* to non-neurodegenerative *N. kadleci* (Aim 2) or vice versa (Aim 3), from AD human patients into *N. kadleci* (Aim 4) or specific microbes or metabolites into *N. furzeri* (Aim 5). Then, I will assess AD markers to determine the effect of the transplantations using a histological approach. Ultimately, we hope to identify the causal role of the gut microbiota in the onset of killifish neurodegeneration and AD. Specific outstanding questions are formulated as the following 5 aims:

1) Can microbiota transplantation from young to aged *Nothobranchius furzeri* delay or prevent neurodegeneration?

2) Can microbiota transplantation from neurodegenerative killifish (*Nothobranchius furzeri*) to non-neurodegenerative killifish (i.e. *Nothobranchius kadleci*) induce neurodegeneration?

3) Can microbiota transplantation from non-neurodegenerative killifish (*Nothobranchius kadleci)* to neurodegenerative killifish (i.e. *Nothobranchius furzeri*) alleviate neurodegeneration?

4) Can microbiota transplantation from Alzheimer’s disease patients into non-neurodegenerative killifish (*Nothobranchius kadleci*) induce neurodegeneration?

5) Can the transplantation of specific microbes/metabolites) to neurodegenerative killifish (*Nothobranchius furzeri*) alleviate neurodegeneration?

1.1.3 Scientifically justified statement that the purpose of the experiment cannot be achieved using methods or processes (e.g. cell cultures, isolated organs, etc.) other than animal experiments (Section 7a (2) No. 2 TierSchG)

Explanations [200 words]:

The killifish model was chosen for this proposal due to its neurodegenerative properties. Currently, no other animal models are available that spontaneously develop neurodegeneration as a consequence of aging. In addition, the short life-span of the killifish allows for efficient investigation of age-related phenotypes such as neurodegeneration. The proposed work will investigate natural age-related neurodegeneration, which is a highly complex process that involves numerous cell-types, signaling pathways and environmental factors. Consequently, there is currently no non-animal based model available that can be used to study such a complex process.

1.1.4.2 Scientifically justified statement that the desired test result is not yet sufficiently known

Explanations [1/2 page]:

Gut dysbiosis, a condition in which microbial homeostasis is disturbed, has been suggested to causally affect Alzheimer’s disease (AD) (Kim et al., 2020). However, the mechanisms by which gut dysbiosis influence neurodegeneration remains largely unknown, hampering the development of treatment options for patients suffering AD disease. Although effort is being made to understand how gut dysbiosis affects AD, the current animal models largely rely on transgenic induction of Aβ42, limiting research into the causal effects of gut dysbiosis on the onset of Aβ42 aggregation and AD. Here, I propose to characterize the causal effects of gut dysbiosis on neurodegeneration using the African turquoise killifish (*Nothobranchius furzeri*) (Matsui et al., 2019), and another closely related killifish species (i.e. *Nothobranchius kadleci*).

1.1.5.1 Intended animal species, justification for the choice of animal species, age, possibly weight and gender (Section 31 Paragraph 1 Sentence 2 No. 1c TierSchVersV)). Description of the lines and their designation according to the international nomenclature

- If necessary, attach the "Final assessment of genetically modified breeding lines" annex -

Explanations [~200 words]:

This project is aimed at characterizing the effects of microbiota transplantation on age-related neurodegeneration in the brains of killifish. The relevance of the project stems in the fact that this vertebrate species offers the unique opportunity to study, in a very short time for a vertebrate (i.e. 3 months), the effects of environmental manipulations that can affect neurodegeneration. Indeed, recent findings have proven that the intestinal microbiota has a key role in modulating neurodegenerative phenotypes in mice models of neurodegeneration. However, these mouse models rely on the transgenic induction of neurodegeneration, rather than resulting spontaneously as a consequence of ageing. Killifish have recently been shown to spontaneously neurodegenerate with ageing, as seen in human Alzheimer’s/Parkinson’s patients. Therefore, using the short-lived, naturally neurodegenerating killifish model, we are in a unique position to ask whether altering the intestinal microbiota can affect the natural onset of neurodegeneration. The age of the fish used for the experiments differ per sub-question and will be further explained in the experimental design (section 1.1.5.2.).

**1.1.5.2 Planned number and justification for the number of animals including information on biometric planning (Section 31 Paragraph 1 Sentence 2 No. 1c TierSchVersV)**

**- - If necessary, attach the "Statistical report" annex -**

**- - If necessary, use the attachment form "Information on biometric planning" -**

**Test and control groups F) Explanations [2 pages]:**

In the proposed work, microbiota from different donors will be transplanted into killifish showing either robust (*N. furzeri*, stain ZMZ1001) or reduced (i.e. *N. kadleci*, strain (NK430)) neurodegenerative symptoms. Detailed experimental design will be discussed per sub-question. Afterwards, more details will be given on various techniques employed in the experimental design.

**Sub question 1: Can microbiota transplantation from young to aged *Nothobranchius furzeri* delay or prevent neurodegeneration?**

Donors

Microbiota will be transferred that is obtained from either 6 week old (“young”, group 3) or 12 week old (“aged”, group 4) *N. furzeri* fish.

Acceptors

Microbiota will be transferred into 12 week old (“aged”, groups 3-4) *N. furzeri* fish. In addition, in groups 6 and 7, another transplant at 19 weeks of age will be performed to increase the potential beneficial effect of the treatment on improvement of cognitive function at time of the tissue isolation (when the fish are 26 weeks old).

Rationale timepoints

For a “young” donor, week 6 was chosen as this is the time point at which the used fish species reach full sexual maturity. We will validate the sexual maturation before isolating the gut microbiota by looking for signs of sexual maturity (coloration in males, fat belly in females).

For the “aged” acceptor group, 12 weeks was chosen because preliminary data shows that neurodegenerative symptoms start appearing around this time.

For the “old” age group from which tissue will be isolated, 26 weeks was selected as a time-point. At this time-point, the killifish start displaying signs of cognitive decline (Terzibasi et al., 2008), suggestive of an advanced neurodegenerative state. Working with this time point, we will be able to address the effect of the treatment on behavioral decline.

Experimental overview

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Group 1**  - untreated fish  - n = 30 | **Group 2**  - antibiotic treated fish  - n = 30 | **Group 3**  - antibiotic treated fish  - receiving young fish microbiota  - n = 30 | **Group 4**  - antibiotic treated fish  - receiving aged fish microbiota  - n = 30 |
| **Day 1** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | - | Recolonization with young donor microbiota | Recolonization with aged donor microbiota |
| **Week 7** | - | - | - | - |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | - | - | - |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Group 5**  - antibiotic treated fish  - n = 30 | **Group 6**  - antibiotic treated fish  - receiving young fish microbiota  - n = 30 | **Group 7**  - antibiotic treated fish  - receiving aged fish microbiota  - n = 30 |
| **Day 1** | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | Recolonization with young donor microbiota | Recolonization with aged donor microbiota |
| **Week 7** | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | Recolonization with young donor microbiota | Recolonization with aged donor microbiota |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

Total fish needed

Neurodegeneration is a multimodal process including the loss of neurons, protein aggregation and an increase in non-neuronal cell types. Preliminary data suggest that individual variation in neurodegeneration between fish is profound (~25%). According to our power analysis, 30 individuals per group would be sufficient to detect enough between-group variation when aging leads to 25% change in the neurodegeneration signal (e.g. from histological analysis), even accounting for large within-group variation in neurodegeneration signal. As published before by the host lab (Smith et al., 2017), one donor fish is required to provide microbiota for two donations.

Based on this analysis, the total amount of fish needed is:

Group 1: 30 fish

Group 2: 30 fish

Group 3: 30 acceptor fish, 30 donor fish (young)

Group 4: 30 acceptor fish, 30 donor fish (aged)

Group 5: 30 fish

Group 6: 30 acceptor fish, 30 donor fish (young)

Group 7: 30 acceptor fish, 30 donor fish (aged)

Total: 11 x 30 fish = **330 fish.**

**Contingencies**

1. **In case no significant effects are found of microbiota transplantations between young and old *Nothobranchius furzeri* (Sub question 1) at all, sub-questions 2, 3, 4 and 5 will not be carried out.**
2. **Based on the results obtained for sub-question 1, we will determine whether one transplantation event (groups 2, 3, 4) or two transplantation events (groups 5,6,7) is more efficient at reducing neurodegeneration in killifish. The most efficient strategy (resulting in the biggest difference and/or the smallest standard variation) will be employed in sub-questions 2, 3, 4 and 5.**
3. **In case the learning assessment did not yield any significant results, this part of the design will be abandoned for sub-questions 2, 3, 4 and 5.**

**Sub question 2: Can microbiota transplantation from neurodegenerative killifish (*Nothobranchius furzeri*) to non-neurodegenerative killifish (i.e. *Nothobranchius kadleci*) induce neurodegeneration?**

Donors

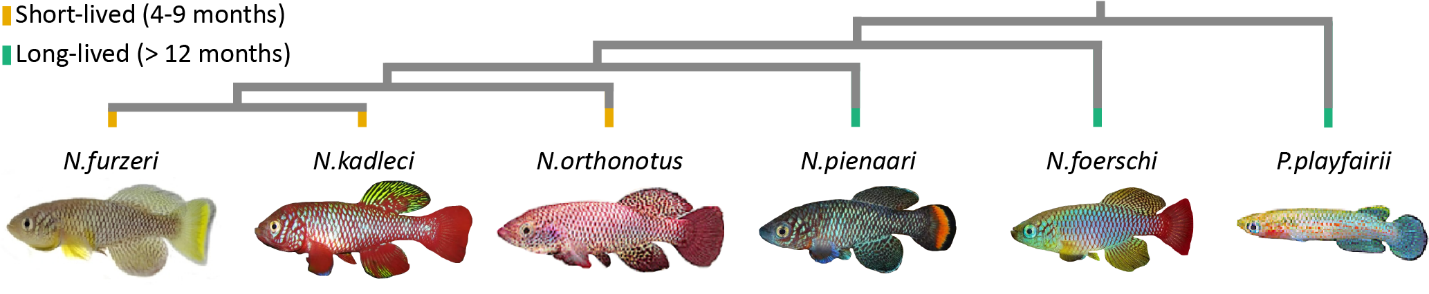
Microbiota will be transferred that is obtained from 26-month old N. furzeri fish.

Acceptors

To address the causal role of gut microbiota during killifish neurodegeneration, we will transplant the gut microbiota from neurodegenerative killifish (*Nothobranchius furzeri*) into a closely related killifish species that shows reduced symptoms of neurodegeneration. Potentially, and under current investigation, is *Nothobranchius kadleci*. However, the species used for this transplantation experiment will be determined by histological comparison of multiple species for which the tissues are already available, including *Nothobranchius furzeri*, *Nothobranchius kadleci*, *Nothobranchius orthonotus*, *Nothobranchius pienaari*, *Nothobranchius foerschi* and *Pachypanchax playfairii* (**Figure 1**). As acceptor species, the most closely related species will be chosen that shows reduced signs of neurodegeneration compared to *N. furzeri*. From this point onwards, *N. kadleci* will form the placeholder species in the experimental design, but might be replaced with a better suited species following the analysis described above.

**Contingencies**

1. **In case no suitable species is identified (showing significantly reduced neurodegenerative symptoms when compared to *N. furzeri*), sub-questions 2, 3 and 4 will not be carried out.**



**Figure 1. Phylogenetic tree of potential acceptor species.**

Rationale timepoints

For the donor group, we chose 26-month old fish (“old”) as at this age, *Nothobranchius furzeri* display robust signs of neurodegeneration including loss of neurons, protein aggregation in the brain and cognitive decline (Matsui et al., 2019; Terzibasi et al., 2008).

Experimental overview

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Group 1**  - untreated *N. kadleci*  - n = 30 | **Group 2**  - antibiotic treated *N. kadleci*  - n = 30 | **Group 3**  - antibiotic treated *N. kadleci*  - receiving old *N. furzeri* donor microbiota  - n = 30 | **Group 4**  - antibiotic treated *N. kadleci*  - receiving with old *N. kadleci* fish microbiota  - n = 30 |
| **Day 1** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | - | Recolonization with old *N. furzeri* donor microbiota | Recolonization with old *N. kadleci* fish microbiota |
| **Week 7** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | - | Recolonization with old *N. furzeri* donor microbiota | Recolonization with old *N. kadleci* fish microbiota |
| **Week 8** | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning |
| **Week 11** | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

Total fish needed

Neurodegeneration is a multimodal process including the loss of neurons, protein aggregation and an increase in non-neuronal cell types. Preliminary data suggest that individual variation in neurodegeneration between fish is profound (~25%). According to our power analysis, 30 individuals per group would be sufficient to detect enough between-group variation when aging leads to 25% change in the neurodegeneration signal (e.g. from histological analysis), even accounting for large within-group variation in neurodegeneration signal. As published before by the host lab (Smith et al., 2017), one donor fish is required to provide microbiota for two donations.

Based on this analysis, the total amount of fish needed is:

Group 1: 30 fish (*N. kadleci*)

Group 2: 30 fish (*N. kadleci*)

Group 3: 30 acceptor fish (*N. kadleci*), 30 donor fish (*N. furzeri*)

Group 4: 30 acceptor fish (*N. kadleci*), 30 donor fish (*N. kadleci*)

Total: 6 x 30 fish = **180 fish.**

**Sub question 3:** **Can microbiota transplantation from non-neurodegenerative killifish (i.e. *Nothobranchius kadleci*) to neurodegenerative killifish (i.e. *Nothobranchius furzeri*) alleviate neurodegeneration?**

Donors

Microbiota will be transferred which are obtained from 12-week old (aged) donor fish which show reduced neurodegenerative symptoms (i.e. *N. kadleci*). The selection of the exact species which will be used for this purpose, is described in the experimental design of sub-question 2.

Acceptors

Acceptors of the microbiota transplantation are neurodegenerative killifish (*N. furzeri*). We will address whether the transplantation can alleviate neurodegenerative symptoms in these fish.

Rationale timepoints

For the “aged” acceptor group, 12 weeks was chosen because preliminary data shows that neurodegenerative symptoms start appearing around this time in *N. furzeri*.

Experimental overview

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Group 1**  - untreated acceptor fish  - n = 30 | **Group 2**  - antibiotic treated acceptor fish  - n = 30 | **Group 3**  - antibiotic treated acceptor fish  - receiving aged *N. kadleci* donor microbiota  - n = 30 | **Group 4**  - antibiotic treated acceptor fish  - receiving with *N. furzeri* donor microbiota  - n = 30 |
| **Day 1** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | - | Recolonization with aged (*N. kadleci*) donor microbiota | Recolonization with aged (*N. furzeri*) donor microbiota |
| **Week 7** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | - | Recolonization with aged (*N. kadleci*) donor microbiota | Recolonization with aged (*N. furzeri*) donor microbiota |
| **Week 8** | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning |
| **Week 11** | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

Total fish needed

Neurodegeneration is a multimodal process including the loss of neurons, protein aggregation and an increase in non-neuronal cell types. Preliminary data suggest that individual variation in neurodegeneration between fish is profound (~25%). According to our power analysis, 30 individuals per group would be sufficient to detect enough between-group variation when aging leads to 25% change in the neurodegeneration signal (e.g. from histological analysis), even accounting for large within-group variation in neurodegeneration signal. As published before by the host lab (Smith et al., 2017), one donor fish is required to provide microbiota for two donations.

Based on this analysis, the total amount of fish needed is:

Group 1: 30 fish (*N. furzeri*)

Group 2: 30 fish (*N. furzeri*)

Group 3: 30 acceptor fish, 30 donor fish (*N. kadleci*)

Group 4: 30 acceptor fish, 30 donor fish (*N. furzeri*)

Total: 6 x 30 fish = **180 fish.**

**Sub question 4:** **Can microbiota transplantation from Alzheimer’s disease patients into non-neurodegenerative killifish induce neurodegeneration?**

Donors

Microbiota will be transferred that is obtained from the stool of Alzheimer’s disease (AD) (or potentially Parkinson’s disease (PD)) patients, or healthy control humans. These samples are available to us via collaboration with Prof. Dr. Ullrich Wüllner from the DZNE in Bonn.

Acceptors

To address the causal role of gut microbiota from AD/PD patients, we will transplant this microbiota into a killifish species (i.e. *N. kadleci*) that show only limited symptoms of neurodegeneration.

Rationale timepoints

For the “aged” acceptor group, 12 weeks was chosen because preliminary data shows that neurodegenerative symptoms start appearing around this time in *N. furzeri*, which is a closely related species to that receiving the transplant.

Experimental overview

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Group 1**  - untreated  *N. kadleci*  - n = 30 | **Group 2**  - antibiotic treated *N. kadleci*  - n = 30 | **Group 3**  - antibiotic treated *N. kadleci*  - receiving old *N. furzeri* donor microbiota  - n = 30 | **Group 4**  - antibiotic treated *N. kadleci*  - receiving with old *N. kadleci* fish microbiota  - n = 30 |
| **Day 1** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | - | Recolonization with AD/PD patient microbiota | Recolonization with control microbiota from healthy humans |
| **Week 7** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | - | Recolonization with AD/PD patient microbiota | Recolonization with control microbiota from healthy humans |
| **Week 8** | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning |
| **Week 11** | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

Total fish needed

Neurodegeneration is a multimodal process including the loss of neurons, protein aggregation and an increase in non-neuronal cell types. Preliminary data suggest that individual variation in neurodegeneration between fish is profound (~25%). According to our power analysis, 30 individuals per group would be sufficient to detect enough between-group variation when aging leads to 25% change in the neurodegeneration signal (e.g. from histological analysis), even accounting for large within-group variation in neurodegeneration signal. As published before by the host lab (Smith et al., 2017), one donor fish is required to provide microbiota for two donations.

Based on this analysis, the total amount of fish needed is:

Group 1: 30 fish (*N. kadleci*)

Group 2: 30 fish (*N. kadleci*)

Group 3: 30 acceptor fish (*N. kadleci*)

Group 4: 30 acceptor fish (*N. kadleci*)

Total: 4 x 30 fish = **120 fish**

**Sub question 5:** **Can the transplantation of specific microbes/metabolites to neurodegenerative killifish (*Nothobranchius furzeri*) alleviate neurodegeneration?**

Donors

Results obtained from the previous 4 sub questions, as well as developments throughout the field, might result in the identification of potential neurodegeneration-inhibiting microbes or metabolites. Here, I will investigate whether these (to be determined factors) can alleviate neurodegeneration in *Nothobranchius furzeri*. As a control, aged microbiota from *N. furzeri* will be transplanted.

Acceptors

We will transplant specific microbes/metabolites into aged *N. furzeri*.

Rationale timepoints

For the “aged” acceptor group, 12 weeks was chosen because preliminary data shows that neurodegenerative symptoms start appearing around this time in *N. furzeri*.

Experimental overview

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Group 1**  - untreated acceptor fish  - n = 30 | **Group 2**  - antibiotic treated acceptor fish  - n = 30 | **Group 3**  - antibiotic treated acceptor fish  - receiving specific microbes/metabolites  - n = 30 | **Group 4**  - antibiotic treated acceptor fish  - receiving with *N. furzeri* donor microbiota  - n = 30 |
| **Day 1** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Day 2**  (acceptors are now 12 weeks old) | - | - | Recolonization with specific microbes/metabolites | Recolonization with aged (*N. furzeri*) donor microbiota |
| **Week 7** | - | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) | Antibiotic treatment (24 hours) |
| **Week 7 + 1 day**  (acceptor age: 19 weeks) | - | - | Recolonization with specific microbes/metabolites | Recolonization with aged (*N. furzeri*) donor microbiota |
| **Week 8** | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning | Active Avoidance conditioning |
| **Week 11** | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning | Memory assessment + Active Avoidance conditioning |
| **Week 14**  (acceptor age: 26 weeks) | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation | Behavioral testing, euthanization and tissue isolation |

Total fish needed

Neurodegeneration is a multimodal process including the loss of neurons, protein aggregation and an increase in non-neuronal cell types. Preliminary data suggest that individual variation in neurodegeneration between fish is profound (~25%). According to our power analysis, 30 individuals per group would be sufficient to detect enough between-group variation when aging leads to 25% change in the neurodegeneration signal (e.g. from histological analysis), even accounting for large within-group variation in neurodegeneration signal. As published before by the host lab (Smith et al., 2017), one donor fish is required to provide microbiota for two donations.

Based on this analysis, the total amount of fish needed is:

Group 1: 30 acceptor fish (*N. furzeri*)

Group 2: 30 acceptor fish (*N. furzeri*)

Group 3: 30 acceptor fish (*N. furzeri*)

Group 4: 30 acceptor fish (*N. furzeri*), 30 donor fish (*N. furzeri*)

Total: 5 x 30 fish = **150 fish**

**Total amount of fish needed:**

Sub question 1: 330

Sub question 2: 180

Sub question 3: 180

Sub question 4: 120

Sub question 5: 150

**Total = 960 fish** (*N. kadleci* = 300 fish, *N furzeri* = 510 fish)

1.2.2 Description of the keeping conditions and the preparation of the animals for the experiment (Section 8 Paragraph 1 Sentence 2 No. 5 TierSchG)

Explanations [1/2 page]:

The animals are kept under standard husbandry conditions and are checked for their condition individually at least once a day by direct visual inspection. In addition, the fish rooms are regularly inspected by the responsible official veterinarian and by FLI veterinarians. In addition, the housing conditions and the functionality of the systems used for housing are regularly checked by the nursing staff. This ensures that no avoidable pain, suffering or harm is inflicted on the animals. The animals are fed with bloodworms twice a day and depending on their age. The *Nothobranchius* husbandry consists of the following rooms: FLI2 S.003 (injection systems / incubation of eggs), S.004 (breeding), S.005 / 006 (experimental housing), S.007 (quarantine), FLI6 E.03 (experimental posture). The fish for this experimental project are bred in S.004 and kept in S.005 / 006. The animals remain in individual housing for the experiment. Before any experiment fish are sacrificed as explained in 1.2.4.

Housing conditions: water temperature 28 ° C (target: 28 ° C), day / night rhythm: 12 h / 12 h, pH value: 6.5-7.8 (target: 7.3), nitrite content : <0.1 mg / l, nitrate content: <50 mg / l.

All experimental systems are operated with permanent water circulation. The water is cleaned using a biofilter and irradiated with UV light in order to minimize pathogens and algae growth. The water treatment takes place in a VE water system in FLI2 and a reverse osmosis system in FLI6. In storage tanks, the water is salted with sea salt (conductivity 2.5-2.8 mS / cm). We will use static tanks for breeding. There is a weekly water change of 50%.

1.2.3 Description of hygiene management J)

Hygiene status of the test animals / animal husbandry:

Explanations [100 words]:

Only trained personnel (transponder, key) have access to all rooms. Access is via a 1-chamber drying lock. Before entering the room, body-covering protective clothing (gloves, changing shoes or using overcoats) is mandatory.

Hygiene monitoring:

Explanations [100 words]:

Hygiene monitoring is carried out through quarterly examinations of pre-filters and post-filters sentinel fish in the individual holding areas by an external company using PCR analyzes for fish pathogens (bacteria, endo- and ectoparasites, viruses), as well as histopathological examinations. In addition, the biofilter is analyzed in every system in the same cycle. In addition, a summative health certificate is drawn up for the individual housing areas.

|  |  |
| --- | --- |
| **1.2.4** | **Describe the practical protocol for all procedures and treatments for each respective experimental group in both type and length including anaesthesia; provide a detailed description of all pr****ocedures and their scheduled administration (Art. 17 in connection with Art. 31 Sect. 1 Clause 2 No. 1d TierSchVersV)** |

**Antibiotic treatment and microbiota recolonization**

To allow for effective colonization of the transplanted microbiota, antibiotic treatment has to be performed. For each group of fish treated with antibiotics, fish will be housed in a single 2.8L tank filled with autoclaved tank water plus antibiotic for 24 hours. Recipient fish will be treated with a combination of Vancomycin (0.01 g/L), Metronidazole (0.5 g/L), Neomycin (0.5 g/L) and Ampicillin (0.5 g/L) to diminish the resident bacterial community, as described in (Smith et al., 2017). During the antibiotics treatment, the water will not flow and fish will be housed in still water. Importantly, these fish are not river fish, and live in stagnant water and for this reason they do not need continuously flowing water for survival. After 12 hours from the beginning of the experiment, 50% of the tank water will be replaced with fresh tank water and the same dose of antibiotic. After antibiotic treatment, acceptor fish will be housed for 24 hours in a new tank with autoclaved tank water, the water flow will be switched off, and the intestinal content of young and old donor fish, respectively, will be added to the tanks. To prevent any harm derived from absence of circulating water, a 50% water change will be con-ducted 12 hours after the start of the treatment in treated fish. During this phase, fish from all groups will not be fed.

**Verfahren am Versuchsende (Euthanasia at the end of the experiment)**

Fische werden durch eine Überdosis Tricain (1 g/l) getötet. Die Fische werden hierzu in ein mit Tricain versetztes Becken überführt. Innerhalb von 5 min tritt der Tod ein. Zusätzlich wird an-schließend eine Dekapitation zur Sicherstellung des Todes durchgeführt (gemäß SOP/Arbeitsanweisung des FLI („Euthanasie mit Tricaine“)). Bezugsquelle für das Tricain ist die tierärztliche Hausapotheke.

Alternativ können die Tiere durch die per Ausnahmegenehmigung genehmigte „Rapid Chilling Methode“ getötet werden. Dafür werden Fische in 0-4°C kaltes Wasser überführt ohne dabei in direkten Kontakt mit Eis zu kommen. Die Fische werden je nach Alter für einen definierten Zeit-raum im eiskalten Wasser euthanasiert, gemäß der SOP/Arbeitsanweisung des FLI („Euthanasie mittels Rapid Chilling“).

**Microbiota isolation**

First, we will euthanize fish as described above. Then, we their intestines will be extracted. Intestines from donor fish will be finely minced and mixed. After mixing, they will be weighed and aliquotted in the appropriate amount of tubes reflecting the amount of doses needed during the recolonization stage.

**Health monitoring**

Fish that are part of the experimental design which have received antibiotic treatment will be checked for health status twice a day after feeding.

**Behavioral testing**

We will test the decline in learning capacity of the treated fish in a behavioral test as described in the protocol for Active Avoidance, which was previously used in publications by the host lab (Terzibasi et al., 2008; Valenzano et al., 2006) (**Figure 2**). A tank (38 3 23 3 18 cm) was divided in two by a hurdle with a rectangular hole (3 3 3 cm) (**Figure 2A**). The two compartments are wedged-shaped to funnel the fish through the hurdle. The tank is filled with water from the home tank and the fish are left to acclimate for 15 min before starting the test. Then, the conditioned stimulus (red light) is delivered in the compartment where the fish is present, then followed by an adverse stimulus (a plastic stick whirling or air bubbling in the compartment). The fish tend to respond to the disturbance by moving to the other compartment. The aim of the test is to detect the acquisition of a strategy to escape the adverse stimulus by crossing the hurdle upon presentation of the conditioned stimulus. **Figure 2B** depicts a scheme of the temporal structure of the test. The conditioned stimulus lasts for 30 s. If the fish does not move to the other compartment after 15 s, the adverse stimulus is delivered for 15 s. The fish moves to the other compartment, rests for 30 s, and then the cycle is repeated. If the fish crosses the hurdle within 15 s after onset of the red light (i.e., before administration of the adverse stimulus) the trial is scored as a ‘‘success,’’ otherwise it is scored as ‘‘failure.’’



**Figure 2. Protocol for Active Avoidance in *N. furzeri*, from Valenzano et al. Current Biology, 2006.**

As part of the proposed work, we will perform the above described test right before the fish are sacrificed for downstream analysis. The test will indicate whether the neurodegenerative state is correlated with a decline in learning capacity, as is the case in human patients suffering neurodegenerative disorders.

To connect the behavioral findings with the downstream histological analysis, we will prepare a small sub-condition that allows c-Fos staining. In short, c-Fos marks active neurons in the brain, including those active during learning (Bullitt, 1990). Using c-Fos, we will ask: in the fish that show reduced learning capacity, is the brain region responsible for this process (c-Fos+) displaying signs of neurodegeneration on histological level.

To answer this question, we need to determine which brain region is responsible for the conditioning of our fish during the learning test. As a negative control, we will not perform the active avoidance conditioning on 5 *N. furzeri* fish (Sub-question 1, experimental group 1, untreated control fish) as well as on 5 *N. kadleci* fish (Sub-question 2, experimental group 1, untreated control fish). Comparison of conditioned (25 fish) and non-conditioned (5 fish) groups will indicate the brain region that contains neurons that are activated (c-Fos+) during the conditioning.

**Downstream analysis**

Brain tissue will be isolated and prepared for histological analysis to assess neurodegeneration. Gut tissue will be isolated and cut in half. One half will be used for the extraction of microbiota and subjected to 16S-profiling. The other half will be prepared for histological analysis.

1.3.1 Scientifically justified statement that the expected pain, suffering or damage to the test animals is ethically justifiable with regard to the purpose of the test (Section 7a (2) No. 3 TierSchG)

Explanations [2/3 page]:

The proposed research might lead to the identification of novel substrates that can be targeted to alleviate neurodegeneration in millions of patients. Indeed, Alzheimer’s and Parkinson’s disease affect over 50 million people world-wide today and are predicted to affect over 150 million by 2050 (World Health Organization, 2019). Therefore, the proposed work might have a profound societal impact by improving the quality of life, as well as relieving economic pressure on the health-care system by mitigating the intensive care necessary to support patients suffering neurodegeneration.

The killifish model was chosen for this proposal due to its neurodegenerative properties. Currently, no other animal models are available that spontaneously develop neurodegeneration as a consequence of ageing. In addition, the natural variation in lifespan and age-related symptoms across different killifish populations allows for comparative genomics, which is a valuable tool in investigating the genetic mechanisms underlying age-related neurodegeneration. The proposed work will investigate natural age-related neurodegeneration, which is a highly complex process that involves numerous cell-types, signaling pathways and environmental factors. Consequently, there is currently no ex-vivo model available that can be used to study such a complex process.

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