Killifish as a potential animal model for delirium investigation; comparison of its validity to currently used animal models.

Gudo Rietman S3992349
Supervisor: dr. Monika Trzpis
Second assessor: prof. Jon Laman

Rijksuniversiteit Groningen 22-4-2023

Abstract

Delirium is a common condition among ill elderly people, especially those with some degree of dementia. The acute onset of the disease is linked to a variety of triggers including precipitating and predisposing factors, often including systemic inflammation in the peripheral system. Currently however, the treatment of the underlying cause of delirium, and consequently treatment that would prevent delirium is impossible, as the pathophysiology of this disease is mostly unknown. However, investigation of the pathophysiology of delirium in human is difficult due to multiple reasons including physiological difficulties. Therefore it is inevitable to use animal models in researching delirium. Current research on delirium is being dune using mostly rodents as animal model, however, a promising animal to conduct research in is the killifish due to the fact that it is the shortest-lived vertebrate known to research, making it potentially highly suitable to conduct age-related research in. The primary aim of this thesis was to evaluate killifish as an option for an animal model for delirium. The findings of this thesis conclude that the utilization of killifish as an animal model for delirium research presents a promising avenue for researching delirium. This model affords the potential to serve as a cost-effective alternative to rodent models. However, it is important to note that substantial work still needs to be done in the design and validation of a killifish model that accurately mimics a delirium-like state.

Contents

Introduction

Delirium is perceived as an acute onset of complications involving the functionality of an individual's cognition combined with altered consciousness which can last for a few hours or as long as several weeks or months (Williams et al., 2020). Delirium is a common condition among ill elderly people, especially those with some degree of dementia. The acute onset of the disease is linked to a variety of triggers including precipitating and predisposing factors, often including systemic inflammation in the peripheral system (Cunningham, 2011; Schreuder et al., 2017; Young & Inouye, 2007). In general, patients who develop delirium have a prolonged hospitalisation time, increased cognitive and functional decline, are more often institutionalized, and have a higher risk of mortality compared to patients who do not develop delirium (Han et al., 2022). Due to these complications, delirium is also a substantial burden on healthcare organizations, costing over US$ 164 billion per year in the United States and US$182 billion per year in 18 European countries combined (Fong & Inouye, 2022). Therefore treatment, and preferably prevention, of delirium will not only improve the quality of life among the patients and their relatives but will also help in solving some major socioeconomic issues affecting the whole of society. Patients diagnosed with delirium often deviate from their normal state of arousal, showing either a hyperactive or agitated state or a hypoactive state which can also be described as a quiet state of being. Hyperactive delirium patients have an increased loss of control in motor activity, suffer from restlessness, and wandering. Hypoactive delirium patients show a decrease in speed of actions and activity, have a reduced awareness of their surroundings, and have a decreased amount of speed and speech. There is also a state of arousal in which patients fluctuate between showing features from hypoactive or hyperactive states of being. The previously mentioned situation is known as mixed delirium (Ali & Cascella, 2023; Girard et al., 2018) To summarize, delirium is a state appearing acutely caused by an underlying medical condition. It is characterized by an alteration of attention, consciousness, and cognition, with a reduced ability to focus, sustain or shift attention (Ramírez Echeverría et al., 2023).

Currently however, the treatment of the underlying cause of delirium, and consequently treatment that would prevent delirium is impossible, as the pathophysiology of this disease is mostly unknown. The current approach to delirium is to treat the symptoms. The patients are treated mostly with antipsychotics to calm them down or to pharmacologically control their arousal, including sleeping and eating pattern. Additionally, patients are asked to use their glasses, hearing apparatuses, they are provided with a clock including not only time but also a date. These steps support patients' spatiotemporal orientation and are considered to prevent patients from delirium or at least milden the symptoms and accelerate overcoming delirium. Sometimes, as prevention, antibiotics are prescribed. These treatments, however, do not treat the pathophysiological mechanisms, which are demonstrated by symptoms indicating delirium (Inouye et al., 2014; Neufeld et al., 2016).

Furthermore, the limited knowledge about delirium pathophysiology hinders optimal diagnoses, leading to suboptimal quality of medical care (Dunne et al., 2021). One of the major problems in the diagnosis of delirium is that it expresses itself in a broad spectrum of symptoms, which can significantly vary between patients (Mattison, 2020). These different forms of delirium presentation indicate that there might be different pathophysiological mechanisms underlying this condition. The identification of biomarkers of delirium, i.e. proteins of which altered expression level is associated with delirium, may give some insight into understanding the pathophysiology and could potentially help with recognizing the disease earlier, treating the patients on time. This, in turn, could prevent developing delirium or at least decrease the severity of delirium and minimise complications after the delirious state of the patient (Dunne et al., 2021; Marcantonio et al., 2006). However, investigation of the pathophysiology of delirium in human is difficult due to multiple reasons. Firstly, physiological

difficulties arise when trying to examine delirium in human patients. The disease involves two different areas including the peripheral system and the brain. Researching the brain area is particularly difficult to investigate in human, you cannot take a sample of the brain when the patient is still alive, unless you collect a biopsy however this is hard to obtain for just the purpose of research. Secondly, the inclusion of patients in studies, requires, most often, the signed informed consent of a patient, while patients with delirium can often be mentally incompetent to make such a decision. In this case, their relatives can decide, but these are not always available (Sidhaye & Knoblich, 2021).

Despite the multiple obstacles, multiple studies have been performed with the aim of identification of the biomarkers which are associated with delirium. Currently, at least 30 different biomarkers have been identified but the outcomes of these studies are still ambiguous and even contradictory (Dunne et al., 2021). The unclear outcomes are related, among others, to the diversity of delirium forms, diversity between patients, their variate medical history, medication they have been using, and also to different instruments used for delirium diagnosis. Moreover, the biomarkers have been identified based on associations, and consequently, they cannot be confirmed as causative factors, which are directly and actively involved in the pathophysiology of delirium. Many of these biomarkers have also been identified to be associated with an inflammatory disease or other cognitive impairments, which provides an indication of the relation between delirium and systemic inflammation but does not explain the mechanisms, which lead to delirium. Furthermore, the implication of the knowledge in treatment of delirium is also currently not possible, because simultaneous deactivating or targeting of multiple biomarkers is rather impossible in human. Preferably one common factor, which for instance underlays the altered expression pattern of the identified delirium-associated biomarkers should be identified. Such a finding would enable development of for instance tailored immunotherapy targeting the biomarker, which underlays the pathophysiology of delirium. To decrease the aforementioned diversity between species, and to avoid cofounders, i.e. multiple comorbidities, which elderly patients frequently experience, and also to investigate consequences of inactivation of a potential biomarker and finally, to test the safety of such an intervention, it is inevitable to use animal models.

With all this all this mind / Consequently / Therefore, fundamental studies involving animals are crucial to add to the knowledge on physiology of delirium. Animal experiments have contributed significantly to the development of fundamental understanding of human disease processes (Robinson et al., 2019). One of the major advantages of using animal models over human experiments is that animal models are clones of each other meaning that each individual has the same genome, which solves the problem of interspecies diversity in case of clinical studies in human. Having test subjects with the same genome gives researchers the opportunity to validate their results without having the uncertainty of other factors interfering with the results.

Animals can be manipulated via e.g. surgery or infection to mimic the pathophysiological phenotype of the diseased human. Furthermore, animal models are of great value, especially in age-related studies. Depending on species and strains, animal models have a significantly shorter life span which allows researchers to conduct age-related studies in a timespan of months in the case of rodents, and days in the case of killifish (Schreuder et al., 2017). Using the current knowledge that systemic inflammation can lead to delirium, this trigger has been used to develop mouse models with delirium. The most important interventions aiming to evoke delirium are lipopolysaccharide (LPS) induced delirium, and sepsis-associated delirium induced by cecal ligation and puncture (CLP) procedure (C. Murray et al., 2012; Schreuder et al., 2017).

There are however several limitations when using animals for delirium investigation. One of the major issues, which is common for all animal models, is how to confirm that an animal has actually developed delirium. In human patients, delirium is diagnosed by using validated scores, including the DOSS or 4AT

scores. These scores examine the spatiotemporal orientation of a patient suspected of having delirium, and also his/her cognition, and ability to focus. The patients are asked whether they know where are they, what is the time, and which day of the week it is. Also, cognition and focus of patients are being tested by for instance asking him/her whether he/she can name all the days of a week in a backwards order. In animal models, it is not possible to perform such tests. Although just by making observations, the diseased animals show similar behavioural features within the animal species, they avoid movement, they do not eat, they just quietly lay in their cage and try to save their energy for recuperating from the disease. Therefore, based on just behaviouristic observation of an animal it is impossible to diagnose whether they have delirium. Consequently, in case of diagnosis of delirium in animals, biochemical validation is needed. For this, information on the spatiotemporal expression of different biomarkers, which have been characterised in human, is being used. Specifically, in rodents, in which delirium has been induced, has been validated by conformation that biomarkers typical for delirium in humans have also been present in the rodents upon interventions aiming to evoke delirium (Mattison, 2020; C. Murray et al., 2012).

The primary aim of this thesis is to evaluate killifish as an option for an animal model for delirium. By analysing the current knowledge gained in human and mouse models, the possible advantages and disadvantages of killifish as an animal model for delirium will be determined.

Current understanding of the pathophysiology of delirium

Pathophysiology of delirium, i.e. the molecular mechanisms which lead to the development of this acute cognitive impairment, has extensively been investigated in human and rodents. Systemic inflammation is thought to be one of the major triggers for the development of delirium. This trigger, and consequently delirium, is most often following trauma, surgery, bladder infection or sepsis in elderly, vulnerable patients (Inouye, 2006; Inouye et al., 2014). One the hypothesis proposes a mechanism, in which pathogenetic mechanisms, related to systemic inflammation play crucial role in delirium development. Specifically, different studies indicated that activation of the immune system leading to an increased number of macrophage in blood, blood monocyte activation and consequently, secretion of inflammatory mediators, such as cytokines, and this, in a currently unknown way, can lead to acute brain inflammation (Marcantonio et al., 2006). It is worth noting that under normal and abnormal conditions, cytokines can also be secreted in the central nervous system by neurons, microglia, astrocytes. Moreover peripheral T-cells and also cytokines can cross the so-called blood-brain barrier in some pathological circumstances, and in this way enter the brain (Marcantonio et al., 2006). In order to comprehend the process by which systemic inflammation shifts / enters to the brain, an extensive evaluation of various cytokines and other immune factors has been conducted on biomaterials obtained from patients with and without delirium. Biomaterials primarily refer to blood, which is readily accessible, but also cerebrospinal fluid (CSF) and brain tissues are crucial to be included in the analysis otherwise we cannot know what happens in the brain, while delirium demonstrates processes which take place in the brain (Hall et al., 2018; Kenneth M Murphy et al., 2022). A combination of studies has identified more than 70 biomarkers thought to be involved with delirium. These markers include e.g. pro-inflammatory cytokines, hormones, and neurotransmitters (Hall et al., 2018). Blocking/knocking out one or a combination of a few biomarkers could give researchers the opportunity to discover their involvement in the pathophysiology of delirium. Once their role in pathophysiology of delirium would be known, opportunities for potential treatment and prevention could arise. The problem however is that deletion of these biomarkers in human is rather impossible to achieve due to both ethical and physiological difficulties. Therefore the use of animal models is inevitable.

Delirium research in rodent models

Characteristics of rodent models

Rodent models have proven to be of great value to biomedical research because their features are quite similar to those of the human species. Rodents have been used in research for an extended period of time. They are used for comparative medicine research which is built on the ability to use the knowledge obtained from one species to understand the same processes in other species (Bryda, 2013). The similarities are manifested in the form of both physiology and anatomy. To bring this into perspective, between rodent and human DNA, around 90% of the genetic information is shared (Breschi et al., 2017). However, delirium is a neuroinflammatory disease, so a more relevant question will be if the immune system is similar between rodents and human. Overall the general structure of the immune system between mice and human is quite similar, however, there is a significant difference in the balance of lymphocytes and neutrophils in the adult animal compared to human. Human blood is neutrophil rich (50–70% neutrophils, 30–50% lymphocytes) whereas mouse blood has a significant majority of lymphocytes (75–90% lymphocytes, 10–25% neutrophils) indicating a difference between the functioning of the immune systems (Doeing et al., 2003; Mestas & Hughes, 2004). These immunological differences may introduce/generate the risk of obtaining results that would work in the animal model but are not compatible when translating it to human trials. The use of rodents as an animal model does serve some economic advantages: rodents need fairly little time to become of adult age which allows researchers who are interested in the field of ageing to start their studies swiftly. The C57BL/6 mice is an often used rodent animal model which lives up to 26-30 months (Yanai & Endo, 2021). Another economically favoured quality of rodents is the fact that they are small in proportion to human and thus need little space and resources, keeping maintenance costs reasonably low. The gestation of rodents is short, the time between conception and birth in mice is approximately 20 days, and for rats this is approximately 22 days, producing as many as 14 pups per litter which allow researchers to generate large numbers of individuals in a short period of time (Breschi et al., 2017; S. A. Murray et al., 2010; Reckelhoff et al., 1992). Maintenance of the animals in a lab is expensive, and according to the animal care & use program of the University of Michigan, the rates for animal husbandry care including items such as the purchase of animals and supplies, daily care of animals, and technical assistance the per-diem costs are between 1,11 – 3,39 dollar per day for rats and mice.

Delirium in rodents

Up to now a few animal models for studying delirium have been used. The most prevalently described in the literature models are made in mice. These mice have been made based on the hypothesis that delirium results from systemic inflammation, which in an unknown yet way, leads to the acute central nervous system (CNS) inflammation. As aforementioned, delirium can be induced using LPS injections or by CLP procedure (C. Murray et al., 2012; Schreuder et al., 2017). The introduction of LPS to the peripheral region of a C57BL/6 mouse strain results in a significant elevation of cytokines, which may endure for several months (Hoogland et al., 2015). After the intervention with LPS, measurements of i.e. the cytokines can be used to determine a delirium-like state in the animal This peripheral (systemic) exposure to LPS stimulates microglia, via cytokine release, and elevated levels of other pro-inflammatory factors, such as for instance, cortisol, contribute to the acute brain inflammation. (Hoogland et al., 2015). The delirious state evoked by the CLP procedure involves the puncturing of the cecum to enable the release of faecal matter into the peritoneal cavity, which induces an systemic inflammation and leads to sepsis, brought on by a polymicrobial infection (Toscano et al., 2011).

Despite multiple studies involving mouse models for studying the pathophysiology of delirium, there are some important limitations of such *in vivo* setup. The major limitation, which has not been

overcome till now, is the fact that it is impossible to diagnose either rodents or any other animal in the way humans are diagnosed with delirium, namely with verbally performed anamnesis. It means thus that it is currently impossible to diagnose whether the animal indeed suffers from delirium. The afore-described models have however been validated as models representing delirium by performing biochemical analysis of their biomaterial, including blood, CSF and brain tissue. The validation revealed that the spatiotemporal fingerprint of biomarkers in the blood and CSF, and also molecular as well as histopathological changes in the CNS of the rodents in which delirium was induced, resembled the changes observed in human who had been diagnosed with delirium (MacLullich et al., 2008; Schreuder et al., 2017).

Experimental procedures in rodent models

Besides looking at the biomarkers, the delirium models have also been confirmed by observing cognitive impairment following the delirium like state, which is a common occurrence in approximately 30% of patients who have experienced delirium (Inouye et al., 2014; Mattison, 2020). These tests are performed after the animals recover from their delirious state. During the course of delirium, the animals are too sick to follow tests to examine their cognitive state. Delirium in patients can often lead to neurodegeneration in the brain, which in turn can result in the development of post-delirium dementia. This phenomenon has also been observed in mice, where surviving a systemic inflammation, possibly resulting in delirium, has been shown to cause neurodegeneration and cognitive impairment over the long term, as evidenced by histopathological changes in their brains. Elderly mice injected with LPS and recovering from systemic inflammation were not able to learn and remember the T-maze system, which validates the model since the same occurs in humans (Cunningham, 2011; O'Neill et al., 2021). So, these cognitive assessments aim to ascertain the manifestation of post-delirium side effects in animals, specifically in the form of dementia or chronic neurodegeneration of the CNS, which is not an acute condition.

T-maze test

The study done by C. Murray et al. aimed to assess the working memory of mice in the hippocampal region, specifically 3-8 hours after administering LPS, by using a T-maze task that measured alternation behaviour. The maze was constructed with a guillotine door and two choice arms with a single hole at the end of each arm. The mice were motivated to leave the maze by walking on their tip-toes or paddling through the 2cm deep water. The task involved blocking one arm, forcing the mice to make a left or right turn in a pseudorandom sequence. After making the first turn, the mouse had to alternate and choose the opposite arm to escape from the water. Correct choices led to an escape through a transit tube, while incorrect choices allowed for self-correction. The task was validated and its details are provided in supplementary data (C. Murray et al., 2012). This test is a form of Pavlovian conditioning, meaning that after several trials the healthy mice will develop contextual memory retrieval allowing them to recognize turn to make to escape the maze (Maren & Holt, 2000).

Novel object recognition test

M. Chen et al. performed a study using C57BL/6 mice with LPS-induced delirium-like state. The test is a relatively simple procedure that involves three days: habituation day, training day, and testing day. During training, the mouse explores two identical objects. On the test day, one of the familiar objects is replaced with a novel one. As mice have a natural preference for novelty, if they recognize the familiar object, they will spend most of their time exploring the novel object. Hence, there is no need for positive or negative reinforcement or extended training schedules. Moreover, the ORT can be adapted for different purposes by altering the retention interval to evaluate short-term or long-term memory (M. Chen et al., 2022; Lueptow, 2017). Like the T-maze test, the novel object recognition test

also relies on the ability of the animal to remember certain environmental stimuli. The underlying mechanism in the mouse model regarding the ORT is its innate preference for novelty depending on the ability to recognize objects encountered previously. Because this experimental procedure allows researchers to investigate both long- and short-term memory it can not only be of value to investigate post-delirium cognitive impairment. The ability to distinguish between short-term and long-term memory in this experimental procedure could give researchers context on which areas of the brain might be affected by delirium because both forms of memory involve the activation of different brain areas (Norris, 2017; Robertson, 2002).

Y-maze test

The Y-maze is a useful tool for evaluating short-term memory in mice. To measure spatial working memory, one can assess spontaneous alternation by allowing the mice to explore all three arms of the maze, which capitalizes on the innate tendency of rodents to explore novel areas. An intact working memory, and thus prefrontal cortical function, will be reflected in the mouse's ability to recall previously visited arms and demonstrate a preference for entering a less recently visited arm. Additionally, spatial reference memory, which is associated with the hippocampus, can be evaluated by placing the mice in the Y-maze with one arm blocked during training. Following an inter-trial interval, such as 1 hour, the mouse should be able to remember the previously unexplored arm and exhibit a higher frequency of entry into that arm (Kraeuter et al., 2019).

Despite plenty of advantages related to the rodent models, there are still certain limitations; as aforementioned, the animals are not cheap to maintain in the lab, they have a lifespan of up to two years, and have an immune system that differs substantially from that of human. Exploring to possibility of using different animal models could potentially provide a better alternative for researching delirium.

Behavioural test	Behavioural domain	Procedure	Parameters that can be tested
T-maze test	Learning and memory retention	animal is allowed to explore the maze for a certain period of time.	Latency to enter the target area (s) Number of entries to the target area (%) Time spent in the target area (s)
Novel object recognition test	Object recognition and memory	Animal is allowed to explore the objects for a certain period of time	Time spent on novel object (s) Preference for novel object (%) Distance travelled from novel object (m)
Y-maze test	Spatial recognition and memory	animal is allowed to explore the maze for a certain period of time.	Spontaneous alternation (%) Time spent in novel arm (s) Distance travelled in novel arm (m)
The three chamber task	Spatial recognition and memory	animal is allowed to explore the maze for a certain period of time.	Latency to enter the target area (s) Number of entries to the target area (%) Time spent in the target area (s)

Table 1 Overview of characteristics of experimental procedures to asses cognitive functions in animals (Tan et al., 2022).

Figure 1 Killifish facility UMCG (Gudo Rietman, 2023)

Killifish as an animal model

One of the promising alternatives to rodent models could be the killifish. Killifish have already been use to investigate the pathophysiology of several diseases such as osteoporosis (Butylina et al., 2022b). The fish have also been used to contribute to understand the age- and a-Synuclein-dependent degeneration of dopamine and noradrenaline neurons related to Parkinson's disease (PD) by monitoring a-Synuclein progression and number of dopaminergic and noradrenergic neurons at 1 and 5 months of age (Matsui et al., 2019). Current research does not provide examples of killifish models where a systemic inflammation is induced, therefore zebrafish models will be considered in which this has already been done.

Characteristics of killifish models

There are multiple hallmarks of killifish (*Nothobranchius furzeri*), which make it a suitable candidate for studying ageing related diseases, including delirium. Firstly, the killifish is the shortest-lived vertebrate known to research, with a lifespan of approximately 3-7 months (Butylina et al., 2022a; Cui et al., 2020). There are indications that the ageing of killifish encompasses many hallmarks that have been postulated to contribute to the ageing process in mammals, such as telomere shortening, cellular senescence, and mitochondrial dysfunction (Corral-Debrinski et al., 1992; Graf et al., 2013; Hartmann et al., 2009). Therefore, killifish resembles a true and valuable model for ageing research. The killifish inhabits ephemeral ponds in south-eastern Africa (Platzer & Englert, 2016a). Due to the conditions in the ponds, the killifish has evolved in having a short life-span. Within the timeframe of the pond containing enough water to be habitable, the eggs have to hatch, the young animal has to mature, and the mature animals have to produce new eggs for the next cycle of a habitable pond before it dries out. Also, because of the short lifespan of the killifish, the fish constitute a great potential for studying ageing-related diseases. Secondly, the embryos of the killifish can survive the dry season because they are encased in dry mud where they enter a dormant state also known as diapause which could last for more than one year. In this dormant state, development is completely arrested, until the new rain season provides a new habitable pond for the fish to thrive in (Platzer & Englert, 2016b; Reichard et al., 2015). This dormant state can be advantageous to researchers because by only adding water the embryos start developing, meaning that the embryos can be stored, ready to be used at any time point. Thirdly, practical matters involved with the killifish could be favourable to conducting a research plan. The costs of maintaining the fish are relatively low when compared to for example mouse models. The costs of maintaining one fish one day are approximately 15 cents according to Professor Eugene Berezikov whose research group has initiated and coordinated the establishment of the killifish facility in the University Medical Centre Groningen (Figure 1). Another advantage is that adult killifish breed readily and can produce as many as tens to hundreds a week (Hu & Brunet, 2018; Platzer & Englert, 2016b). This is quite different from mice as they generally produce litters of one to 14 pups. The huge amount of offspring with killifish enables the researchers to obtain a large group of animals on which research can be performed in a short amount of time. Furthermore, killifish embryos are clear, which allows scientists to watch the fertilized eggs grow into fully formed baby fish under a microscope. Their transparency also enables the visualization of fluorescently labelled tissues in transgenic killifish embryos, which, in context of delirium, ie. disease which although taking place in brain, is most possibly initiated in the peripheral system, would be a very relevant feature to follow the course of the inflammation (Hu & Brunet, 2018). Different scientists developed a transparent killifish line through multiplex CRISPR/Cas9 mediated gene inactivation allowing the scientists to also visualize fluorescently labelled tissues in adult killifish (Krug et al., 2023). Unlike zebrafish, turquoise killifish do not require specific conditioning to trigger mating and breeding behaviours (Hu & Brunet, 2018). Unfortunately, there is currently not enough information available to evaluate whether the immune system of killifish is comparable to the human immune system. However, it is known that macrophages and neutrophils

are important components to consider when comparing the immune system of killifish to humans, as they are crucial in all types of inflammatory diseases (Hu & Brunet, 2018). On the other hand, studies have shown that the immune system functions are similar in zebrafish and humans, making zebrafish an excellent model organism for studying inflammation in an in vivo setting (Xie et al., 2021). The immune systems of killifish and zebrafish share some similarities. Studies have shown that zebrafish have a well-developed immune system with similarities to that of mammals, including humans. This includes similar immune cell types and mechanisms for recognizing and responding to pathogens (e.g., bacteria and viruses). While there is limited information available on the immune system of killifish, a recent study has shown that the killifish immune system also involves the same types of immune cells, such as macrophages and neutrophils, which are crucial for inflammatory responses in both fish and mammals (Han et al., 2022; Hu & Brunet, 2018). However, further research is needed to fully understand the similarities and differences between the immune systems of these two fish species.

Experimental steps to induce delirium in killifish

In order to induce a delirium-like state in killifish, it is necessary to evoke a systemic inflammation in the animal. At present, there are no documented cases of systemic inflammation being induced in killifish, therefore procedures performed in zebrafish could be considered to be translated to killifish. When considering what is known to induce a large inflammation in zebrafish, the tail fin amputation model is a prevailing method, involving the partial removal of a zebrafish larva's tail fin. The use of this model has been extensive in exploring crucial elements of the inflammatory reaction, including the roles of particular signalling pathways, the migration of leukocytes, and the interactions among different immune cells. Other models have utilized chemical interventions such as LPS, leukotriene B4 (LTB4), and copper to induce inflammation. Certain chemical-induced models, like the administration of trinitrobenzene sulfonic acid (TNBS), specifically aim to provoke inflammation in the gastrointestinal tract (Xie et al., 2020; Xiong et al., 2022). Given the similarities between zebrafish and killifish, these approaches hold considerable promise for inducing a delirium-like state in killifish.

Experimental procedures for validation of the model and testing cognitive performance in killifish models

To validate that the killifish model is in a similar state as human delirium patients, analyses are necessary, akin to the rodent models, through the examination of biomarkers. Biomarkers can be obtained through blood collection, cytokine measurement, or plasma isolation. Because of the small size of the killifish blood withdrawal can be a challenging procedure, however, Dolfi et al., managed to perform repeated blood withdrawals from individual killifish sampling up to 8 µL depending of different factors such as age, sex, and size (Dolfi et al., 2023). Additionally, determining whether killifish exhibit post-delirium cognitive impairment, similar to rodent models and human delirium patients, is a critical question that needs answering. Experimental procedures must be conducted to investigate post-delirium cognitive impairment. These experimental procedures are currently being used for research in zebrafish, which need to be translated into an experiment suited for killifish (Tan et al., 2022). Table 2 shows an overview of the experimental procedures that could be performed to evaluate cognitive function.

The three-chamber task

The three-chamber task consists of a central chamber flanked by two side chambers, each separated by movable partitions mounted on Plexiglas rails. To aid in position discrimination, dark strips are placed along the rear side of the tank to provide an axis of orientation. The choice chamber is divided into two compartments, which can be opened to allow the fish to swim into either side, then closed once the fish has made its decision. If the fish makes an incorrect choice, it is confined to the end of the choice chamber for a period of 10 seconds as a penalty. After each trial, the fish is returned to the

central start chamber by opening the partition. This experiment utilizes differential reinforcement, whereby incorrect choices are penalized by restricting the fish's swimming space, while correct choices are rewarded by allowing the fish to remain in the full-sized choice chamber (Levin, 2011). So with this experimental procedure, spatial recognition and memory can be tested.

Y-maze test

In the Y-Maze task, the arms are assigned randomly, with one arm designated as the starting arm, always open for fish to explore. Another arm, called the novel arm, is initially blocked during the first trial but left open during the second trial. The remaining arm is always open as well. The neutral zone at the centre of the maze was not analysed. The task involves two trials separated by a training-test interval (TTI) and is used to evaluate the response of fish to novelty. The Y-Maze memory task is a dependable method to assess memory in zebrafish, offering a novel and quick alternative that is not reliant on preference or avoidance behaviour. This task is well-suited for studying memory in this type of fish. (Cognato et al., 2012).

Novel object recognition test

Similar to the Y-maze, which evaluates fish response to novelty, a novel object recognition test is utilized to measure recognition memory. Capatina et al. conducted a test in which a zebrafish was placed in a tank with two red cubes, representing familiar objects, and allowed to explore for 10 minutes. After an hour, one of the red cubes was replaced with a green cube, representing a novel object, and the fish's exploration was recorded. An exploration was defined as coming within 2.5 cm of an object. Recognition memory was assessed by measuring exploratory time and the preference for the novel object (Capatina et al., 2021; Tan et al., 2022).

T-maze

To establish conditioned stimulus (CS) – unconditioned stimulus (US) pairing through repeated exposure, a rectangular tank or maze is commonly used. Chen et al. utilized a T-maze and covered the left and right arms with green and red coloured sleeves to serve as visual cues. One of the arms, designated as the enriched chamber, contained brine shrimp as an appetitive stimulus. The experimental protocol included habituation, training, and probe phases. The authors evaluated memory performance by measuring the latency to enter and time spent in the targeted zone. Other appetitive stimuli that can be used include the sight of conspecifics, an appealing environment with artificial grass and stones, among others (J. Chen et al., 2020; Tan et al., 2022).

Conclusion

Whether killifish would make a suitable animal model to study delirium, several aspects need to be considered. Davidson et al., stated that when choosing a model for research, especially animal models, there are several factors that should be taken into consideration, including: 1) appropriateness as an analog, 2) transferability of information, 3) genetic uniformity of organisms, where applicable, 4) background knowledge of biological properties, 5) cost and availability (Davidson et al., 1987). The appropriateness of killifish as an analog to human is difficult to answer because there is not enough research conducted on killifish to be able to make conclusions about this. However, considering that zebrafish and killifish share biological features and have homology in genetics and tissue structure it is highly plausible that killifish research could also be used analog to human research just as what is currently being done with zebrafish research (Kodera & Matsui, 2022). With this being considered, the transferability of information and genetic uniformity between killifish and human is highly suitable for conducting research related to delirium. Also, practical features of killifish could improve research regarded to delirium compared to the research that has been done till now. Table 2 shows some of the most prominent features of killifish and rodent models covered in this thesis. After evaluation of these

facts, one can conclude that the potential killifish model would be more practical to conduct research on, the costs are lower, killifish mature faster allowing a shorter time of research, and readily availability of new individuals to be tested. Though these promising outcomes, one has to be cautious because killifish as a model is still in early development. There still has to be an excessive background knowledge of biological properties established to validate the findings. Until this validation some of these assumptions are based on speculations provided by the literature.

In conclusion, the utilization of killifish as an animal model for delirium research presents a promising avenue for researching delirium. This model affords the potential to serve as a cost-effective alternative to rodent models. However, it is important to note that substantial work still needs to be done in the design and validation of a killifish model that accurately mimics a delirium-like state.

Characteristics	Rodent	Killifish
Lifespan	26-30 months in C57BL/6 mice	3-7 months
Number of offspring	1-14 per litter	10s-100s per week
Costs of maintenance per day	1,11 – 3,39 dollar	0,15 dollar
Immune system compared to human	Significant difference in balance lymphocytes - neutrophils	Comparable with human
Gestation time	20-22 days	14-21 days
Time to become of adult age	3-6 months	14 days

Table 2 presentation of directly comparable features shared by both rodents and killifish.

References

Ali, M., & Cascella, M. (2023). *ICU Delirium*.

Breschi, A., Gingeras, T. R., & Guigó, R. (2017). Comparative transcriptomics in human and mouse. *Nature Reviews Genetics*, *18*(7), 425–440. https://doi.org/10.1038/nrg.2017.19

Bryda, E. C. (2013). The Mighty Mouse: the impact of rodents on advances in biomedical research. *Missouri Medicine*, *110*(3), 207–211.

Butylina, M., Föger-Samwald, U., Gamsjaeger, S., Wahl-Figlash, K., Kothmayer, M., Paschalis, E. P., Pusch, O., & Pietschmann, P. (2022a). <i>Nothobranchius furzeri,</i> the Turquoise Killifish: A Model of Age-Related Osteoporosis? *Gerontology*, *68*(12), 1415–1427. https://doi.org/10.1159/000524300

Butylina, M., Föger-Samwald, U., Gamsjaeger, S., Wahl-Figlash, K., Kothmayer, M., Paschalis, E. P., Pusch, O., & Pietschmann, P. (2022b). Nothobranchius furzeri, the Turquoise Killifish: A Model of Age-Related Osteoporosis? *Gerontology*, *68*(12), 1415–1427. https://doi.org/10.1159/000524300

Capatina, L., Napoli, E. M., Ruberto, G., & Hritcu, L. (2021). Origanum vulgare ssp. hirtum (Lamiaceae) Essential Oil Prevents Behavioral and Oxidative Stress Changes in the Scopolamine Zebrafish Model. *Molecules (Basel, Switzerland)*, *26*(23). https://doi.org/10.3390/molecules26237085

Chen, J., Fan, R., Wang, Y., Huang, T., Shang, N., He, K., Zhang, P., Zhang, L., Niu, Q., & Zhang, Q. (2020). Progressive impairment of learning and memory in adult zebrafish treated by Al2O3 nanoparticles when in embryos. *Chemosphere*, *254*, 126608. https://doi.org/10.1016/j.chemosphere.2020.126608

Chen, M., Xiao, Y., Zhang, F., Du, J., Zhang, L., Li, Y., Lu, D., Wang, Z., & Wu, B. (2022). Tangeretin prevents cognitive deficit in delirium through activating RORα/γ-E4BP4 axis in mice. *Biochemical Pharmacology*, *205*, 115286. https://doi.org/10.1016/j.bcp.2022.115286

Cognato, G. de P., Bortolotto, J. W., Blazina, A. R., Christoff, R. R., Lara, D. R., Vianna, M. R., & Bonan, C. D. (2012). Y-Maze memory task in zebrafish (Danio rerio): The role of glutamatergic and cholinergic systems on the acquisition and consolidation periods. *Neurobiology of Learning and Memory*, *98*(4), 321–328. https://doi.org/10.1016/j.nlm.2012.09.008

Corral-Debrinski, M., Horton, T., Lott, M. T., Shoffner, J. M., Beal, M. F., & Wallace, D. C. (1992). Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. *Nature Genetics*, *2*(4), 324–329. https://doi.org/10.1038/ng1292-324

Cui, R., Willemsen, D., & Valenzano, D. R. (2020). Nothobranchius furzeri (African Turquoise Killifish). *Trends in Genetics : TIG*, *36*(7), 540–541. https://doi.org/10.1016/j.tig.2020.01.012

Cunningham, C. (2011). Systemic inflammation and delirium: important co-factors in the progression of dementia. *Biochemical Society Transactions*, *39*(4), 945–953. https://doi.org/10.1042/BST0390945

Davidson, M. K., Lindsey, J. R., & Davis, J. K. (1987). Requirements and selection of an animal model. *Israel Journal of Medical Sciences*, *23*(6), 551–555.

Doeing, D. C., Borowicz, J. L., & Crockett, E. T. (2003). Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clinical Pathology*, *3*(1), 3. https://doi.org/10.1186/1472-6890-3-3

Dolfi, L., Ripa, R., Medelbekova, D., Ballhysa, E., Symmons, O., & Antebi, A. (2023). Nonlethal Blood Sampling from the Killifish Nothobranchius furzeri. *Cold Spring Harbor Protocols*. https://doi.org/10.1101/pdb.prot107745

Dunne, S. S., Coffey, J. C., Konje, S., Gasior, S., Clancy, C. C., Gulati, G., Meagher, D., & Dunne, C. P. (2021). Biomarkers in delirium: A systematic review. *Journal of Psychosomatic Research*, *147*, 110530. https://doi.org/10.1016/j.jpsychores.2021.110530

Fong, T. G., & Inouye, S. K. (2022). The inter-relationship between delirium and dementia: the importance of delirium prevention. *Nature Reviews Neurology*, *18*(10), 579–596. https://doi.org/10.1038/s41582-022-00698-7

Girard, T. D., Exline, M. C., Carson, S. S., Hough, C. L., Rock, P., Gong, M. N., Douglas, I. S., Malhotra, A., Owens, R. L., Feinstein, D. J., Khan, B., Pisani, M. A., Hyzy, R. C., Schmidt, G. A., Schweickert, W. D., Hite, R. D., Bowton, D. L., Masica, A. L., Thompson, J. L., … MIND-USA Investigators. (2018). Haloperidol and Ziprasidone for Treatment of Delirium in Critical Illness. *The New England Journal of Medicine*, *379*(26), 2506–2516. https://doi.org/10.1056/NEJMoa1808217

Graf, M., Hartmann, N., Reichwald, K., & Englert, C. (2013). Absence of replicative senescence in cultured cells from the short-lived killifish Nothobranchius furzeri. *Experimental Gerontology*, *48*(1), 17–28. https://doi.org/10.1016/j.exger.2012.02.012

Hall, R. J., Watne, L. O., Cunningham, E., Zetterberg, H., Shenkin, S. D., Wyller, T. B., & MacLullich, A. M. J. (2018). CSF biomarkers in delirium: a systematic review. *International Journal of Geriatric Psychiatry*, *33*(11), 1479–1500. https://doi.org/10.1002/gps.4720

Han, Q. Y. C., Rodrigues, N. G., Klainin-Yobas, P., Haugan, G., & Wu, X. V. (2022). Prevalence, Risk Factors, and Impact of Delirium on Hospitalized Older Adults With Dementia: A Systematic Review and Meta-Analysis. *Journal of the American Medical Directors Association*, *23*(1), 23-32.e27. https://doi.org/10.1016/j.jamda.2021.09.008

Hartmann, N., Reichwald, K., Lechel, A., Graf, M., Kirschner, J., Dorn, A., Terzibasi, E., Wellner, J., Platzer, M., Rudolph, K. L., Cellerino, A., & Englert, C. (2009). Telomeres shorten while Tert expression increases during ageing of the short-lived fish Nothobranchius furzeri. *Mechanisms of Ageing and Development*, *130*(5), 290–296. https://doi.org/10.1016/j.mad.2009.01.003

Hoogland, I. C. M., Houbolt, C., van Westerloo, D. J., van Gool, W. A., & van de Beek, D. (2015). Systemic inflammation and microglial activation: systematic review of animal experiments. *Journal of Neuroinflammation*, *12*, 114. https://doi.org/10.1186/s12974-015-0332-6

Hu, C.-K., & Brunet, A. (2018). The African turquoise killifish: A research organism to study vertebrate aging and diapause. *Aging Cell*, *17*(3), e12757. https://doi.org/10.1111/acel.12757

Inouye, S. K. (2006). Delirium in older persons. *The New England Journal of Medicine*, *354*(11), 1157–1165. https://doi.org/10.1056/NEJMra052321

Inouye, S. K., Westendorp, R. G. J., & Saczynski, J. S. (2014). Delirium in elderly people. *Lancet (London, England)*, *383*(9920), 911–922. https://doi.org/10.1016/S0140-6736(13)60688-1

Kenneth M Murphy, Casey Weaver, & Leslie J Berg. (2022). *Janeway's Immunobiology*.

Kodera, K., & Matsui, H. (2022). Zebrafish, Medaka and Turquoise Killifish for Understanding Human Neurodegenerative/Neurodevelopmental Disorders. *International Journal of Molecular Sciences*, *23*(3). https://doi.org/10.3390/ijms23031399

Kraeuter, A.-K., Guest, P. C., & Sarnyai, Z. (2019). The Y-Maze for Assessment of Spatial Working and Reference Memory in Mice. *Methods in Molecular Biology (Clifton, N.J.)*, *1916*, 105–111. https://doi.org/10.1007/978-1-4939-8994-2_10

Krug, J., Perner, B., Albertz, C., Mörl, H., Hopfenmüller, V. L., & Englert, C. (2023). Generation of a transparent killifish line through multiplex CRISPR/Cas9mediated gene inactivation. *ELife*, *12*. https://doi.org/10.7554/eLife.81549

Levin, E. D. (2011). Zebrafish assessment of cognitive improvement and anxiolysis: filling the gap between *in vitro* and rodent models for drug development. *Revneuro*, *22*(1), 75–84. https://doi.org/10.1515/rns.2011.009

Lueptow, L. M. (2017). Novel Object Recognition Test for the Investigation of Learning and Memory in Mice. *Journal of Visualized Experiments*, *126*. https://doi.org/10.3791/55718

MacLullich, A. M. J., Ferguson, K. J., Miller, T., de Rooij, S. E. J. A., & Cunningham, C. (2008). Unravelling the pathophysiology of delirium: A focus on the role of aberrant stress responses. *Journal of Psychosomatic Research*, *65*(3), 229–238. https://doi.org/10.1016/j.jpsychores.2008.05.019

Marcantonio, E. R., Rudolph, J. L., Culley, D., Crosby, G., Alsop, D., & Inouye, S. K. (2006). Serum biomarkers for delirium. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *61*(12), 1281–1286. https://doi.org/10.1093/gerona/61.12.1281

Maren, S., & Holt, W. (2000). The hippocampus and contextual memory retrieval in Pavlovian conditioning. *Behavioural Brain Research*, *110*(1–2), 97–108. https://doi.org/10.1016/S0166-4328(99)00188-6

Matsui, H., Kenmochi, N., & Namikawa, K. (2019). Age- and α-Synuclein-Dependent Degeneration of Dopamine and Noradrenaline Neurons in the Annual Killifish Nothobranchius furzeri. *Cell Reports*, *26*(7), 1727-1733.e6. https://doi.org/10.1016/j.celrep.2019.01.015

Mattison, M. L. P. (2020). Delirium. *Annals of Internal Medicine*, *173*(7), ITC49–ITC64. https://doi.org/10.7326/AITC202010060

Mestas, J., & Hughes, C. C. W. (2004). Of mice and not men: differences between mouse and human immunology. *Journal of Immunology (Baltimore, Md. : 1950)*, *172*(5), 2731–2738. https://doi.org/10.4049/jimmunol.172.5.2731

Murray, C., Sanderson, D. J., Barkus, C., Deacon, R. M. J., Rawlins, J. N. P., Bannerman, D. M., & Cunningham, C. (2012). Systemic inflammation induces acute working memory deficits in the primed brain: relevance for delirium. *Neurobiology of Aging*, *33*(3), 603-616.e3. https://doi.org/10.1016/j.neurobiolaging.2010.04.002

Murray, S. A., Morgan, J. L., Kane, C., Sharma, Y., Heffner, C. S., Lake, J., & Donahue, L. R. (2010). Mouse gestation length is genetically determined. *PloS One*, *5*(8), e12418. https://doi.org/10.1371/journal.pone.0012418

Neufeld, K. J., Yue, J., Robinson, T. N., Inouye, S. K., & Needham, D. M. (2016). Antipsychotic Medication for Prevention and Treatment of Delirium in Hospitalized Adults: A Systematic

Review and Meta-Analysis. *Journal of the American Geriatrics Society*, *64*(4), 705–714. https://doi.org/10.1111/jgs.14076

Norris, D. (2017). Short-term memory and long-term memory are still different. *Psychological Bulletin*, *143*(9), 992–1009. https://doi.org/10.1037/bul0000108

O'Neill, E., Griffin, É. W., O'Sullivan, R., Murray, C., Ryan, L., Yssel, J., Harkin, A., & Cunningham, C. (2021). Acute neuroinflammation, sickness behavior and working memory responses to acute systemic LPS challenge following noradrenergic lesion in mice. *Brain, Behavior, and Immunity*, *94*, 357–368. https://doi.org/10.1016/j.bbi.2020.12.002

Platzer, M., & Englert, C. (2016a). Nothobranchius furzeri: A Model for Aging Research and More. *Trends in Genetics*, *32*(9), 543–552. https://doi.org/10.1016/j.tig.2016.06.006

Platzer, M., & Englert, C. (2016b). Nothobranchius furzeri: A Model for Aging Research and More. *Trends in Genetics*, *32*(9), 543–552. https://doi.org/10.1016/j.tig.2016.06.006

Ramírez Echeverría, M. de L., Schoo, C., & Paul, M. (2023). *Delirium*.

Reckelhoff, J. F., Yokota, S. D., & Baylis, C. (1992). Renal autoregulation in midterm and late-pregnant rats. *American Journal of Obstetrics and Gynecology*, *166*(5), 1546–1550. https://doi.org/10.1016/0002-9378(92)91632-k

Reichard, M., Cellerino, A., & Valenzano, D. R. (2015). Turquoise killifish. *Current Biology : CB*, *25*(17), R741-2. https://doi.org/10.1016/j.cub.2015.05.009

Robertson, L. T. (2002). Memory and the brain. *Journal of Dental Education*, *66*(1), 30–42.

Robinson, N. B., Krieger, K., Khan, F. M., Huffman, W., Chang, M., Naik, A., Yongle, R., Hameed, I., Krieger, K., Girardi, L. N., & Gaudino, M. (2019). The current state of animal models in research: A review. *International Journal of Surgery (London, England)*, *72*, 9–13. https://doi.org/10.1016/j.ijsu.2019.10.015

Schreuder, L., Eggen, B. J., Biber, K., Schoemaker, R. G., Laman, J. D., & de Rooij, S. E. (2017). Pathophysiological and behavioral effects of systemic inflammation in aged and diseased rodents with relevance to delirium: A systematic review. *Brain, Behavior, and Immunity*, *62*, 362–381. https://doi.org/10.1016/j.bbi.2017.01.010

Sidhaye, J., & Knoblich, J. A. (2021). Brain organoids: an ensemble of bioassays to investigate human neurodevelopment and disease. *Cell Death and Differentiation*, *28*(1), 52–67. https://doi.org/10.1038/s41418-020-0566-4

Tan, J. K., Nazar, F. H., Makpol, S., & Teoh, S. L. (2022). Zebrafish: A Pharmacological Model for Learning and Memory Research. *Molecules (Basel, Switzerland)*, *27*(21). https://doi.org/10.3390/molecules27217374

Toscano, M. G., Ganea, D., & Gamero, A. M. (2011). Cecal ligation puncture procedure. *Journal of Visualized Experiments : JoVE*, *51*. https://doi.org/10.3791/2860

Williams, S. T., Dhesi, J. K., & Partridge, J. S. L. (2020). Distress in delirium: causes, assessment and management. *European Geriatric Medicine*, *11*(1), 63–70. https://doi.org/10.1007/s41999-019-00276-z

Xie, Y., Meijer, A. H., & Schaaf, M. J. M. (2020). Modeling Inflammation in Zebrafish for the Development of Anti-inflammatory Drugs. *Frontiers in Cell and Developmental Biology*, *8*, 620984. https://doi.org/10.3389/fcell.2020.620984

Xiong, Y., Halima, M., Che, X., Zhang, Y., Schaaf, M. J. M., Li, M., Gao, M., Guo, L., Huang, Y., Cui, X., & Wang, M. (2022). Steamed Panax notoginseng and its Saponins Inhibit the Migration and Induce the Apoptosis of Neutrophils in a Zebrafish Tail-Fin Amputation Model. *Frontiers in Pharmacology*, *13*, 946900. https://doi.org/10.3389/fphar.2022.946900

Yanai, S., & Endo, S. (2021). Functional Aging in Male C57BL/6J Mice Across the Life-Span: A Systematic Behavioral Analysis of Motor, Emotional, and Memory Function to Define an Aging Phenotype. *Frontiers in Aging Neuroscience*, *13*, 697621. https://doi.org/10.3389/fnagi.2021.697621

Young, J., & Inouye, S. K. (2007). Delirium in older people. *BMJ (Clinical Research Ed.)*, *334*(7598), 842–846. https://doi.org/10.1136/bmj.39169.706574.AD