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Refinement of zebrafish anaesthesia – a behavioural and mechanistic approach to study memory alterations

Jorge Miguel Custódio Martins Ferreira

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Jorge Miguel Custódio Martins Ferreira

REFINEMENT OF ZEBRAFISH ANAESTHESIA – A BEHAVIOURAL AND MECHANISTIC APPROACH TO STUDY MEMORY ALTERATIONS

Doctoral Thesis in Molecular and Cell Biology;

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Doctor Ana Maria Marques Valentim

Junior Researcher

i3S – Instituto de Investigação e Inovação em Saúde

Doctor Mark Collins

Senior Lecturer

University of Sheffield

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Whether it [all] ends on this insane, chaotic, high-energy jam, or it ends on the most mellow, spiritual, beautiful music imaginable.

You take a deep breath and are like,

"*OK, all right, that's it.*

It's done."

Adapted from Eric Gould

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Balu, meow! *Knocks stuff over*

For the ones that I didn't mention, and I'm sure there's a lot of you. *(Insert your name here)*, thank you!

Contents

Abbreviations

5HT - Serotonin

AC - Alternate Current

AMPA - α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionicacid

BDNF - Brain-Derived Neurotrophic Factor

C - Control Group

CAMKII - Ca2+/Calmodulin-Dependent Protein Kinase II

CN - Control Group Without Revisit

CNS- Central Nervous System

CPA - Conditioned Place Aversion

CPP - Conditioned Place Preference

DC - Direct Current check

EU - European Commission

FDR - False Discovery Rate

FTMS - Fourier Transform Mass Spectrometry

GABA - Glycine And γ-Aminobutyric Acid

GABAA - γ-Aminobutyric Acid Type A

GAPDH - Glyceraldehyde 3-Phosphate Dehydrogenase

GPCRs - G-Protein Coupled Receptors

HCl - Hydrochloric Acid

IQR - Interquartile Range

LTD - Long-Term Depression

LTM – Long-term Memory

LTP - Long-Term Potentiation

MS - Mass Spectrometry

MS222 -Tricaine Methanesulfonate

N - Naiive

NMDA - N-Methyl-D-Aspartate

NS - Nonsignificant

NSF - N-Ethylmaleimide-Sensitive Factor

P/L - Propofol/ Lidocaine

PBS - Phosphate-Buffered Saline

POCD - Post-operative Cognitive Dysfunction

PSD - Postsynaptic Density Proteins

PSU - Power Source Unit

PVDF - Polyvinylidene Fluoride

RAB3aa - Ras-Related Protein Rab-3Aa

RT - Room Temperature

SD - Standard Deviation

SNAP - Synaptosomal-Associated Protein

SNARE - SNAP Receptor

STM - Short-Term Memory

STXBP1 - Syntaxin Binding Protein 1

TBST - Tris-Buffered Saline with Tween

WM - Working Memory

Abstract

This work aims to provide a comprehensive analysis of anaesthesia for the animal model zebrafish, by evaluating the commonly used anaesthetic and possible alternatives focusing on a holistic approach to assess the impact of these on animal welfare and research outcomes.

The perception of drugs as aversive by animals can affect their behaviour and physiology, inducing distress and reducing welfare. In Chapter 1, animals treated with MS222, propofol/lidocaine, and clove oil showed different behavioural profiles compared to a positive control for aversion (HCl), contrary to etomidate. Furthermore, all anaesthetic treatments, except etomidate, presented similar levels of cortisol. However, there is a hypothesis that anaesthetics did not induce low aversion, but the animals forgot anaesthetic exposure due to their potential amnesic properties. Therefore, to assess memory performance, the authors created a one-trial inhibitory avoidance task with accessibility and cost in mind. Chapter 2 provides a validated electric shock task in zebrafish using a custom-designed apparatus with refined parameters for the electrical shock. In Chapter 3, this apparatus and electrical shock were used, and the use of as positive control for memory loss was used (the NMDA antagonist MK-801), validating the task for memory evaluation for the TU and AB strains. Chapter 4 proposed investigating the effects of anaesthetics MS222 anaesthetics and propofol/lidocaine (P/L) on memory, using the validated task, and synaptic activity in zebrafish. The results showed that the P/L combination caused memory impairment in a fear conditioning task, but not MS222. Mass spectrometry analysis of the synaptosome fraction showed that the decrease of specific proteins, such as NSFb and STXBP1b, may explain the cognitive impairment observed in the P/L group. In addition, the decrease in two components of the SNARE complex induced by the P/L may lead to problems in the acquisition/consolidation of the shock memory, with the added difficulty that recall may also be impaired by the alteration of synaptic vesicle docking and fusion, and SNARE recycling via STXBP1b and NSFb, respectively.

This thesis highlights the importance of considering aversion when selecting anaesthetic protocols to ensure animal welfare compliance and provide scientifically sound data. It showed that none of the anaesthetics tested are completely innocuous, but etomidate has more side effects. P/L should be carefully used when memory is evaluated in research, but it provides amnesia of stressful events. Additionally, MS222 does seem to be an adequate anaesthetic for daily use in our facilities. Finally, it shows an important effect of MS222 and P/L anaesthetic protocols, on the main synaptic exocytosis-related proteins, which opens the door to further research to try and clarify the role of anaesthesia on Post Operative Cognitive Disfunction.

Sumário

Este trabalho tem como objectivo fornecer uma análise abrangente da anestesia para o modelo animal peixe-zebra, avaliando os anestésicos habitualmente utilizados e as alternativas possíveis, centrando-se numa abordagem holística para avaliar o impacto destes no bem-estar dos animais e nos resultados da investigação.

A percepção dos fármacos como aversivos pelos animais pode afectar o seu comportamento e fisiologia, induzindo stress e reduzindo o seu bem-estar. No Capítulo 1, os animais tratados com MS222, propofol/lidocaína, óleo de cravo apresentaram perfis comportamentais diferentes em comparação com um controlo positivo para aversão (HCl), ao contrário do etomidato. Além disso, todos os tratamentos anestésicos, excepto o etomidato, apresentaram níveis semelhantes de cortisol. No entanto, existe a hipótese de que os anestésicos não induziram baixa aversão, mas os animais esqueceram a exposição ao anestésico devido às suas potenciais propriedades amnésicas. Por conseguinte, para avaliar o desempenho da memória, os autores criaram uma tarefa *One-trial inhibitory avoidance task*, tendo em conta a acessibilidade e o custo. O capítulo 2 apresenta uma tarefa validada utilizando um aparelho feito à medida em que o choque eléctrico produz aversão. No Capítulo 3, foram utilizados este aparelho, o choque eléctrico e foi utilizado um controlo positivo para a perda de memória (o antagonista NMDA MK-801), validando a tarefa de avaliação da memória para as estirpes TU e AB. O Capítulo 4 propôe investigar os efeitos dos anestésicos MS222 e propofol/lidocaína (P/L) na memória e na actividade sináptica do peixe-zebra. Os resultados mostraram que a combinação P/L provocou uma diminuição da memória numa tarefa de condicionamento do medo, mas não o MS222. A análise por espectrometria de massa da fracção de sinaptossomas mostrou que a diminuição de proteínas específicas, como a NSFb e a STXBP1b, pode explicar a deficiência cognitiva observada no grupo P/L. Além disso, a diminuição de dois componentes do complexo SNARE induzida pelo P/L pode levar a problemas na aquisição/consolidação da memória do choque, com a dificuldade acrescida de que o processo de recordar também pode ser prejudicado pela alteração do acoplamento e fusão de vesículas sinápticas e da reciclagem de SNARE através de STXBP1b e NSFb, respectivamente.

Esta tese salienta a importância de considerar a aversão ao seleccionar protocolos anestésicos para garantir a conformidade com o bem-estar dos animais e fornecer dados cientificamente sólidos. Demonstrou que nenhum dos anestésicos testados é completamente inócuo, mas o etomidato tem mais efeitos secundários. O P/L deve ser utilizado com cuidado quando a memória é avaliada em investigação, pois proporciona amnésia de acontecimentos stressantes recentes. Além disso, o MS222 parece ser um

anestésico adequado para uso quotidiano nas nossas instalações. Por fim, o estudo mostra um efeito importante dos protocolos anestésicos MS222 e P/L nas principais proteínas relacionadas com a exocitose sináptica, o que abre a porta a novas investigações para tentar esclarecer o papel da anestesia na Disfunção Cognitiva Pós-Operatória.

General Introduction

Every year, millions of individuals are being anaesthetised for clinical or research purposes. Clinically, anaesthesia is used whenever there is a need for sedating the patient or to make them unconscious for a surgery. In an animal research context, anaesthesia is one of the main procedures being performed daily [1], using sedation to immobilize the animals for imaging, or using deeper anaesthesia for performing painful procedures or for any other procedures that requires the relieve of animals' potential pain or distress. With this, it is expected that a significantly large number of non-human animals and humans to be in contact with anaesthesia at least once in their lives.

Although anaesthesia is routinely used with success, the process is still not fully understood. There are still risks not considered/mitigated, such as aversion to the anaesthetic and/or cognitive impairment that has the potential to affect many individuals. In humans, at least 40% of the patients over 60 years subjected to anaesthesia suffer from immediate cognitive deficits, with 10% of them reporting those deficits 3 months after anaesthesia [2]. This data is related to non-cardiac surgery patients as it is known that cardiac surgery by itself has an impact in cognition [3]. For animals, it has been shown that anaesthesia affects both spatial and non-spatial memory in mice [4, 5] and working memory and cognitive flexibility alterations in zebrafish [6]; this species also showed signs of aversion to anaesthetics [7-10].

Anaesthesia

Theoretically, anaesthesia causes a reversible intoxication that puts an individual into a state of unconsciousness, and then allows full recovery. The induction of hypnosis (loss of consciousness), analgesia (lack of sensation that also blunts autonomic responses), and muscle relaxation generally results from the full induction of anaesthesia on what is called the triad of anaesthesia (Figure 1) [11]. With unconsciousness, amnesia may also be attained.

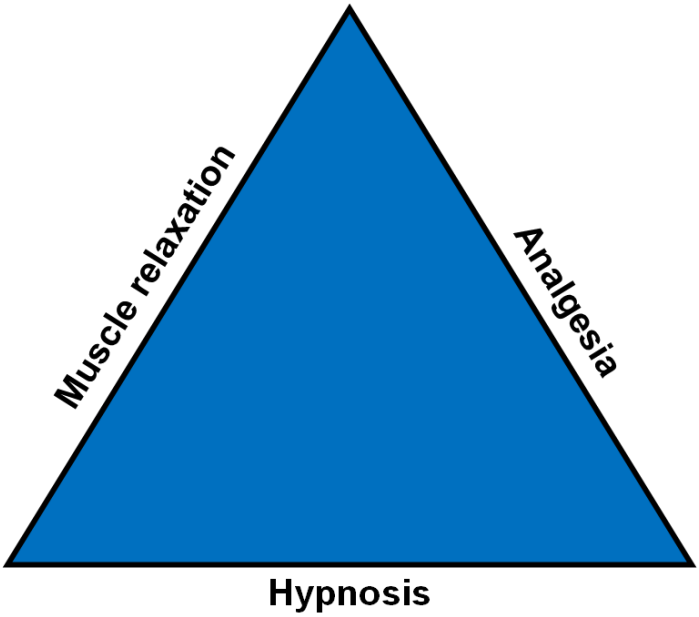

Figure 1: Triangle of anaesthesia including the three major effects that an anaesthetic can induce.

Anaesthesia starts with a phase called induction, where the individual is exposed to the anaesthetic agent, and increases its activity (excitement phase), which is followed by a loss of consciousness/equilibrium. This is followed by the anaesthetic maintenance phase, that involves extending the previous stage without harming the individual. If a long period of anaesthetic maintenance is needed, the anaesthetic concentration must be decreased, and oxygenation provided. To recover from continuous anaesthesia, the anaesthetic administration is stopped and/or sometimes a drug is administered to reverse the anaesthetic effects; afterwards, the individual regains consciousness, returning later to its normal state [12, 13]. If the necessary precautions are not taken, and the anaesthesia lasted for too long, too much anaesthetic is provided or oxygenation is not properly balanced, death may occur due to cardiovascular and respiratory depression. Usually, the depth of anaesthesia is directly proportional to the concentration of the anaesthetic used, and it is critical to regulate it carefully to prevent potentially fatal or harmful overdose, as well as inadequate anaesthesia that may fail to achieve unconsciousness and pain relief (Table 1).

The post anaesthetic period is also a delicate phase, where, to avoid mortality or others comorbidities, general good care and cautious handling are essential, such as providing oxygenation, good water quality, and analgesia when required [14-16].

Stage	Level of anaesthesia	Description
I	**Excitation**	**Anxiety, increase physical activity, rapid breathing rate**
II	**Light anaesthesia**	**Loss of equilibrium, decreased breathing rate, decreased muscle tone, loss of pain perception**
IIa	**Loss of balance**	
IIb	**Myorelaxation**	
IIc	**Loss of pain perception**	
III	**Deep anaesthesia**	**No physical activity, few breathing movements**
IV	**Overdose**	**No physical activity, no breathing, death**

Table 1: Anaesthesia stages for fish and description of the visual cues determining each stage based on Köhler & Valentim [17, 18].

Potential general mechanisms behind anaesthesia

Recent research has increased our understanding of the mechanisms behind general anaesthesia. Numerous scientists have investigated the behavioural effects of general anaesthetics and how they interact with different biochemical targets and areas of the brain. It is interesting to note that there is a strong correlation between the functional regions that general anaesthetics target and the binding sites of ion channel receptors, [11, 19].

Neurotransmitters play a critical role in intercellular communication across the brain. They can either be excitatory, like glutamate and acetylcholine, or inhibitory, like glycine and γ-aminobutyric acid (GABA). They are released in response to electrical signals. Excitatory neurotransmitters allow an influx of sodium ions into the cell, which results in depolarization, to exceed the firing threshold and relay the stimulus. Inhibitory neurotransmitters reduce postsynaptic activity regulating the ion flow through their interactions with ion channel receptors.

The physiological action of general anaesthetics and the range of behavioural reactions they cause are significantly influenced by ion channels. These ion channels, glycine, nicotinic acetylcholine, GABAA, and N-methyl-D-aspartate (NMDA) receptors are all vulnerable to general anaesthetics [11, 19, 20]. Anaesthetics may also have an impact on sodium, potassium, and calcium voltage-gated channels [21]. According to their particular interactions with ion channels, general anaesthetics can either increase or reduce the

activation of inhibitory postsynaptic channels or excitatory synaptic channels [20], controlling the synapse communication.

As any other drug/ treatment, to be widely used, anaesthetics must possess several qualities to be deemed clinical effective and safe. They must be highly soluble in any matrix (e.g. blood, water in the case of fish), potent, have a significant safety margin, induce complete recovery, and produce a diverse range of anaesthesia depths [7]. Safety is often related to hemodynamic alterations, but other side effects have been reported, as anaesthesia interfere with the neurotransmitter system, as referred.

General anaesthesia affects brain function at all levels, including neuronal membranes, receptors, ion channels, neurotransmitters, cerebral blood flow, and metabolism. This can lead to impairment on cognitive and psychomotor performance in the days following general anaesthesia and surgery and such impairment has been named as Post-operative Cognitive Dysfunction (POCD) [22].

POCD is defined as a transient and/or durable subtle dysfunction in one or more cognitive functions, of which memory is typically affected and this alteration may last from a few days to as long as few months [23]. This disorder is currently being investigated in both animal models and humans [22]. Krenk et al. [24] point out in their review that POCD can arise at any age, but tends to last longer and to affect everyday life and the return to work of aged individuals**.** It has been reported that, in older individuals, POCD affects 1 in 3 patients during the first week after the intervention and 1 in 10 patients up until 3 months after [2].

This disfunction has also been described as potentially linked to neuroinflammation, which initiates the process with microglial activity [25] Glial activation and the consequent release of proinflammatory cytokines within the hippocampus interfere with cognitive function as evidenced by abnormal memory and learning and/or inability to develop long-term potentiation [26]. It has been suggested that proinflammatory cytokines could play a role in the development of cognitive decline that may acutely follow surgery or anaesthetic insult [22]. In animal models, there is a similar effect in animals that undergo surgery [27-30], but the results of animals treated with anaesthesia only, are contradictory in the literature [4, 27, 31-34], probably due to methodological differences (type of behavioural task, anaesthesia duration, animals' age). Nevertheless, some studies showed animals that without surgical trauma, still have altered neuroinflammatory parameters [34]. In this situation, the stress of being manipulated and tested may be the neuroinflammatory trigger [35] that promotes this deleterious effect when combined with anaesthesia, which is modulated by age.

The mechanism of how surgery and anaesthesia cause side effects has been a topic of research for at least the past two decades [36]. In the early 2000s, it was demonstrated that general anaesthesia produces long-lasting impairment in the ability of rats to acquire and perform a spatial memory task with increased effect in aged rats; performance that remained impaired for at least 2 weeks after general anaesthesia [32]. Other studies showed that a low concentration of isoflurane caused significantly poorer performance in mice when compared to control or high isoflurane concentration groups [5, 37, 38]. As these results cannot be explained by the pharmacokinetics of the drugs involved [22], these effects on memory could result from altered synaptic plasticity [39].

Apart from the referred clinical implications of this amnesic effect, this may also produce a great impact in research using animals; if anaesthesia produces effects that are not being considered, bias is introduced in the results wasting valuable resources. In addition, anaesthesia by itself can generate a behaviourally mediated aversion in animals as they cannot comprehend the process which they are going through. This can lead to stress which may trigger neuroinflammation, as previously referred. This stress may happen due to the nausea or ‘emergence delirium’ associated with recovery from inhalant anaesthesia, that has been seen in rodents [40], or animals may experience aversion due to an uncontrollable loss of balance and consciousness.

In the interest of the animal welfare and the quality of the research outcomes, it is essential to understand these side effects in the different animal models and to choose the best anaesthetic for each situation.

3Rs and the importance of refinement

Around 9 million animals are used every year in Europe alone for research purposes [41]. Mice are the most use animals, followed by fish, and then rats (Figure 2). This means that a great number of different procedures is done daily, ranging from surgery to sampling, many of which typically require the use of anaesthesia. With this, there is a wide range of opportunities to apply the 3Rs and improve the quality of research being done with animal models.

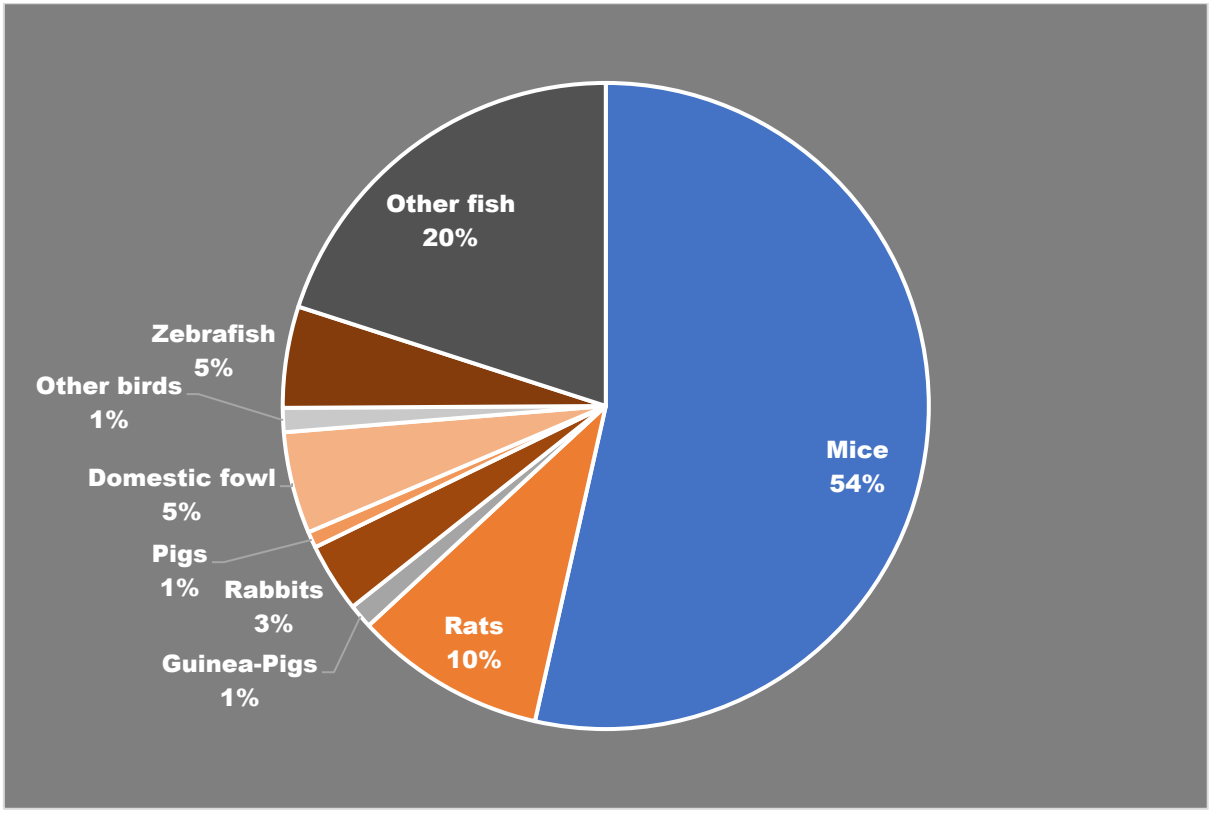

Figure 2: Representation of the distribution of animals used, at least 0.5%, in research. Data from the 2020 European commission report [41].

In 1959, WMS Russell and RL Burch published the concepts of replacement, reduction, and refinement in the book The Principles of Humane Experimental Technique [42]. Meanwhile, the concepts description was updated [43]:

Replacement minimizes research animal distress by substituting animals that can experience distress with insentient material that is incapable of feeling anything and therefore cannot experience distress. Reduction minimizes research animal distress by decreasing the number of animals that can experience distress. Refinement is, by definition, diminution or elimination of distress. This definition of refinement is not intended to suggest that replacement and reduction have a different aim. Refinement is presented as a distinct method of removing inhumanity because it focuses on the actual conduct of research, and on how sentient research animals are treated.

The 3R's need a consistent framework of studies, especially in emergent animal models, as there is a bigger knowledge gap there. One example of that is the zebrafish, which belongs to the second group of animals most used in research and represents around 20% of all the fish used in Europe [41].

Zebrafish and its importance as an animal model

Zebrafish (*Danio rerio*) has been increasing in popularity and use since the 80's, when it was introduced in laboratory. This dramatic increase in use can be seen by the exponent number of papers being published using this animal model. (Figure 3).

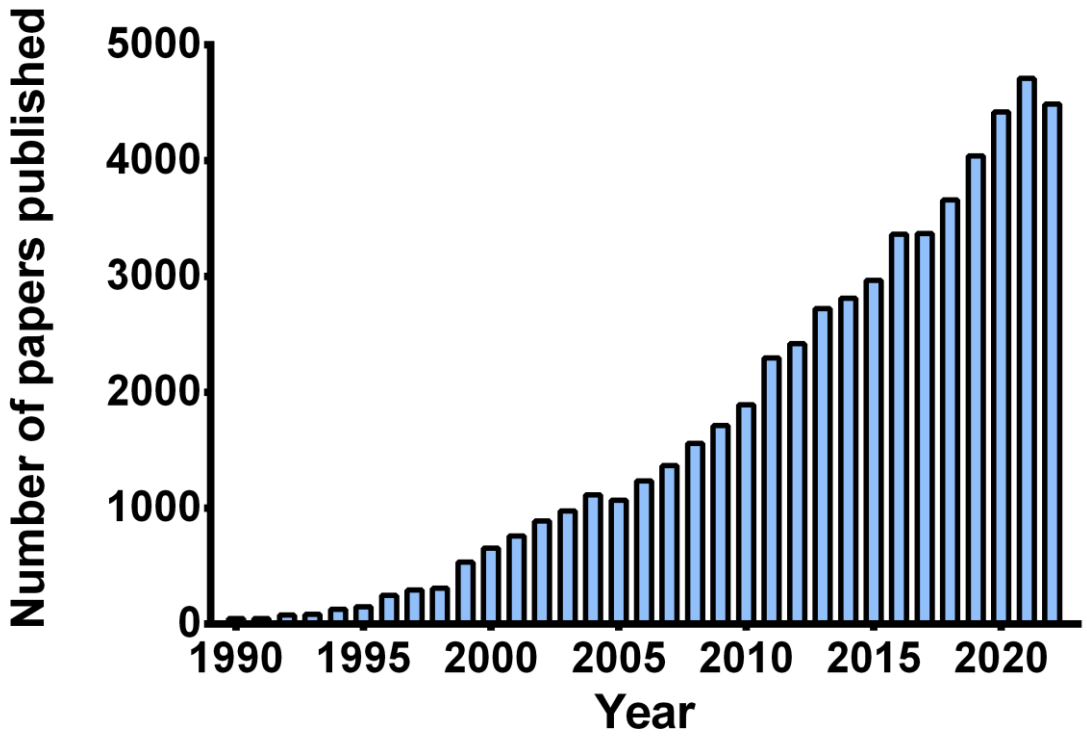

Figure 3: Number of papers published throughout the years using "zebrafish" as a keyword for searching in Pubmed database.

This increase in use is because zebrafish are very cost-efficient, easy to breed and breeds all year round with high fecundity (200–300 eggs per female per spawning every other day) [44], have a short lifecycle (up to 2 years, with sexual maturity around 3 months), a small size allowing to be housed in large numbers in a relatively small space, while being easily maintained and used in laboratory settings [7, 45]. Plus, the zebrafish genome is also well characterized and fully sequenced, revealing a close parallel with mammals [46]. External fertilization and transparency of embryos and larvae increase their value in genetic manipulations, protein tracking, developmental and toxicological studies [47]. This model is also used other research fields, for example to study learning and memory [48, 49], development [50], and regeneration [51]. Despite this increasing use, this has not been accompanied by a full understanding of the animal welfare, leading to the need of refinement of several techniques, as, for example, anaesthesia protocols.

Anaesthesia in zebrafish

Zebrafish are capable of nociception and pain perception [52], thus it is mandatory to reduce distress and pain caused by procedures using analgesia and anaesthesia. This is required in procedures such as surgery, fin clipping for genotyping purposes, and when immobility is needed, for example for imaging [53].

The most common way to induced anaesthesia in zebrafish is by immersion. Here we place the fish in the anaesthetic solution that will then be absorbed through the gills, entering the blood circulation, similarly to the anaesthetic inhalation in terrestrial animals [14]. Then, when returned to clean water, they will regain consciousness. The level of anaesthesia is monitored by recording gill ventilation rates, maintenance of equilibrium (upright position), and reflex responses (e.g., response to tail pinch). The major issue with establishing suitable anaesthetic protocols is that they need to be refined depending of the purpose of the experiment (type of procedure and anaesthesia depth required), the potential influence of the drug in the outcomes, and the stage of development and genetic background of the animal [9]. Taking all of this into account, experiments can be better designed to evaluate and characterise the different possible anaesthetic protocols. With this in mind, we decided to study the standard anaesthetic MS222, propofol and lidocaine in combination, etomidate, and clove oil as they all have been used before in fish successfully, but scarce data is available regarding side effects such as aversion and memory alterations in zebrafish.

MS222

Tricaine methanesulfonate (also known as MS222) is the most popular sedative and anaesthetic for zebrafish acting as a muscle relaxant that blocks sodium (Na^+) channels. By preventing Na^+ from entering the nerve, MS222 lowers the excitability of the neuron membrane, suppressing the nervous system [21, 54]. Although is called a local anaesthetic, when absorbed by the fish gills and skin in an anaesthetic bath, MS222 has a systemic effect [54].

Given that MS222 is available as a water-soluble powder that is acidic in solution, it may cause irritation in the skin and mucous membranes of the fish, increasing the risk of acidosis on fish's plasma. For this reason, it is usually prepared in a buffered solution adding, for example, sodium bicarbonate or sodium hydroxide [55] to MS222. Irrespective of buffering or not, zebrafish exposed to this anaesthetic may respond with tachyventilation before righting reflex, body, and opercular movements cease [56]. In addition, it has been brought up that it could be inducing neuromuscular blockade instead of loss of consciousness, which would cause stress [7].

With the possibility of MS222 not being the best anaesthetic for zebrafish due to the reasons stated above, there is a need for studying more in depth the possible stress effect and if needed, to propose an alternative to MS222. More work needs to be produced to improve fish anaesthesia in terms of both therapeutic effectiveness and animal welfare. Little has previously been done to evaluate the possibility that these chemicals could cause fish suffering [7].

Propofol

Propofol (2,6-diisopropylphenol) has sedative-hypnotic properties [57]. Frequently, it is administered as an anaesthetic drug for induction of anaesthesia in humans and in veterinary practice [58]. In addition, it is also used during maintenance of anaesthesia, for sedation and short duration general anaesthesia outside the operating room. In contrast to MS222, propofol is mainly applied in mammalian species [59]. It provides a reliable, rapid and smooth induction of anaesthesia, adequate hypnosis, with minimal suppression of vital organ functions [58]. It is also possible that it provides some analgesia at high doses [58] which comes with an increased risk of death. Propofol is a short-acting, rapidly metabolized agent, which is characterized by a lack of cumulative effects and a rapid recovery after its administration, either by effective doses or by continuous infusion [58]. The presumed main mechanism of action of propofol is through the potentiation of the chloride current mediated through the γ-aminobutyric acid type A (GABAA) receptor complex [11]. Moreover, recovery is observed to be rapid, uncomplicated, and complete.

Lidocaine

Lidocaine (2-(diethylamino)-N-(2, 6-dimethylphenyl) acetimide) was used for many years as a local anaesthetic agent and it is also an antiarrhythmic drug [60] in mammals, inducing cardiac depressant effects. It is used for local, topical, and regional intravenous block, peripheral nerve block, and spinal and epidural anaesthesia in humans [11]. Nevertheless, it has been used in fish, and its analgesic and/ or anaesthetic effects have been demonstrated in zebrafish [61], and other fish [62-64]. In small fish such as zebrafish, lidocaine is placed in the water, providing general anaesthesia by acting systemically [61, 65]. It is relatively cheap, easy to obtain, and, in most fish, it has a good safety margin [14].

Its primary mode of action is the blockage of voltage-gated Na+ channels [21], same as MS222, and it can prevent or lessen the perception of pain by lowering the peak currents of Na+ channels and speeding up their deactivation process [66].

Propofol/lidocaine combination

In clinical settings, anaesthesia rarely consists of one drug, but rather a combination of drugs to achieve desired levels of hypnosis, analgesia, and muscle relaxation [11]. This increases safety levels for drugs that present issues at high doses. So, our group proposed a new protocol with the potential to be used in zebrafish combining propofol and lidocaine. When propofol is combined with lidocaine both doses can be decreased inducing safe balanced anaesthesia [65]. The application of this concept leads to a safe protocol with analgesia and potentially fewer side effects [9].

Propofol/lidocaine has proven to be effective for procedures requiring a rapid loss of consciousness and analgesia gain (such as the trimming of zebrafish tail fin), but MS222 is preferable when a quick recovery is required [65]. Consequently, this combination has been evaluated in terms of clinical indicators [65], but little is known about its effect on animal welfare.

Clove oil

Clove oil is highly lipophilic and is absorbed through the skin and gills rapidly, entering the bloodstream, crossing the blood–brain barrier, and inducing loss of consciousness [67] acting systemically. It is relatively inexpensive and is a topical anaesthetic cleared for use as an anaesthetic for a variety of fish [68]. This agent is widely available, has a low cost and wide range of efficacy [69]. However, it needs to be solubilized in an organic solvent such as ethanol prior to anaesthesia, which increases the workload, while introducing one more chemical compound [17,18]. Its main active ingredient is eugenol (4-allyl-2-methoxyphenol). Eugenol is rapidly absorbed and metabolized, and it is almost completely excreted in the urine within 24 h with no apparent ill effects in fish [67].

More recent studies have also shown eugenol as an effective alternative to MS222 for the anaesthesia of a variety of fish species [70].

Etomidate

Etomidate [*R* -1-(1-ethylphenyl)imidazole-5-ethyl ester] is a non-barbiturate hypnotic agent that was introduced into clinical practice in 1972 and is primarily used as an anaesthetic in human and small animal veterinary medicine. For fish, it was first described in 1982 as effective anaesthetic [71]. It is water soluble in an acidic solution and becomes lipid soluble at a physiological pH. The mechanism of this effect of etomidate is not fully understood; however, it is known to penetrate the blood-brain barrier rapidly. It is postulated that its

hypnotic effect can be attributed partly to its action on the GABAminergic system in the CNS of mammals, being a GABA receptor agonist [72].

Etomidate does not provide analgesia, but it is a relatively quick acting anaesthetic agent [12].

Anaesthesia side effects

This section pictures a brief description of often disregarded side effects. In addition to the cardiovascular and respiratory alterations, being chemicals, anaesthetics may also interfere with the animals' behaviour, for example, inducing aversion, stress or anxiety and altering cognitive function as previously described.

Aversion

As discussed previously, behaviours linked to aversion may occur during anaesthesia induction. Exposure to certain chemicals during anaesthesia induction may trigger avoidance responses due to the discomfort experienced in their scales or gills. These organs are very sensitive and act as a barrier between the fish and the surrounding water, making them particularly vulnerable to the chemical effects. Such aversive behavioural responses show a similar profile to fear and anxiety responses.

Fear is a functional emotion with an evolutionary origin connected to survival where the species developed coping mechanisms, strategies, and behaviours when presented with a threat [73]. Depending on the environmental conditions, fish may freeze, hide, or, alternatively, escape the hostile environment to deal with threats [74]. Anxiety may overlap with fear emotion, as both are aversive states where the usual response to a threat is escaping. However, anxiety is part of the animal profile, that can be exhibited even when no threats are present, but only the probability of occurring something negative [45]. The escape response to an aversive stimulus has been widely used in research in areas such as, neurophysiology [75], and behavioural ecology [76]. With this, we can infer that behaviour can be used to understand more in depth what is happening with our fish as emotions modulates behaviour.

Memory and learning

Learning and memory are biological processes common to all animals. From single-cell organisms through the nematode and Drosophila to higher-order vertebrates, learning and

memory allow the organism to plastically respond to the changing environment. This mechanism speeds up adaptation that otherwise would take many generations if we would wait for natural selection to play a role in adapting to the ever-changing environment. Apart from basic science, it is clinically relevant to study learning and memory as there are numerous diseases that impair learning and memory [77]. According to conservative estimates, vertebrate genomes harbour approximately 20000 to 35000 genes and at least 60% of these genes are expressed in the brain [78-80]. Many of these genes are believed to be linked to neural plasticity [81]. Briefly, one may expect that with over 10,000 genes indirectly involved in learning and memory processes, only a few hundred have been so far discovered to be linked to this phenomenon [82].

Learning a task and adapting the information are processes where we must store the information and form a memory; later, to perform the learned task, we must recall the memory of that information. For example, when in a T-maze, fish learns in which arm the food is given, then it must store and remember the location of that arm. In the next trials, the memory of where the rewarded arm was is recalled. Therefore, inherent memory processes can be viewed as a product of learning, or vice versa, i. e., the individuals only learn because they recall formed memories where new information can be added and so on. So, we can discuss if these processes are closely intertwined or if they are only one phenomenon. Nevertheless, learning is often described as the process in which memory is acquired, whereas memory is often defined as the process whereby the acquired information is consolidated, maintained, stored, and recalled [82].

Memory classification

Memories can be classified according to their:

- content: declarative/ explicit [83, 84], defined as the conscious storage and recollection of data, and procedural/ implicit [84, 85], defined as unconscious recollection such as fears or habit performed tasks [86].
- nature: archival (Short-Term Memory, Long-Term Memory) as opposed to transient, moment-to-moment (Working Memory) [87]. This memory lasts seconds to few minutes [87, 88].
- duration: Long (LTM) and Short Term Memory (STM). [87] Although there is ongoing discussion about when STM ends and LTM begins, STM is characterized by memories lasting some minutes, while LTM take several hours [89, 90] [91].

Declarative memories are not immediately established as long-term memories (LTMs); this process takes 3–6 h and involves a sequence of specific molecular processes [89, 90]. In

order for us to be able to separate STM from LTM, in 1993 [91], researchers experimentally showed that the cyproheptadine (a non-specific 5HT antagonist) blocks the short- (2–6 min) but not the long-lasting (24 h) facilitation of a monosynaptic response induced by serotonin (5HT). Showing that there is indeed a separation between STM and LTM as it is possible to affect the former while not affecting the later.

Immediate memory lasting seconds or a few minutes is identified as WM [87, 88]. Unlike STM and LTM which are like an off-line system (using computers as an analogy) where we store information for future use, WM is not an archive of individual memories but rather a supervisor/manager of momentarily cognitive events that may relate to information that is being stored or will be stored [92] . Contrarily to what was believed in many of the early experiments, memory measured in mammals 1 minute or so after acquisition is, in fact, WM and not STM. WM is a type of memory primarily dependent on the electrical activity of cells of the prefrontal cortex in humans linked to that of other regions [92]. It persists only while this electrical activity persists (an online system made up of temporary files).

Memory and learning-related proteins

Although the full process of how memory works is not fully understood, we can say that learning experiences trigger a cascade of events changing the brain which will ultimately encode a memory. Research has been focused on neuronal plasticity, where proteins are modified and synthetized [93], synapses created and genes expressed. Then, that event will be encoded in short-term memory and, if deemed favourable, encoded into long-term memory. Synaptic plasticity is critical for this to happen, as it provides the possibility to strengthen, weaken or destroy formed synaptic connections, being them deemed favourable or not. This mechanism can be influenced by external events such as anaesthesia causing memory impairment [94]. This modulation of synaptic connections in response to prior neuronal activities and environmental events is mediated by numerous molecular mechanisms many of which poorly understood. Some of these are autonomous consequences of synaptic activation that involve mechanisms more or less contained within themselves and intrinsic to a given activated synapse [95]. Chemical synapses are specialized junctions between nervous system cells or with non-neuronal cells that allow signals to travel to the target [96]. These are organized around a synaptic cleft formed by the highly differentiated presynaptic element and post-synaptic element. The presynaptic membrane is covered with synaptic vesicles filled with various neurotransmitters ready to be released into the cleft. This release happens in response to the arrival of an action potential with its associated wave of elevated calcium, activating the many voltage-gated calcium channels present [95].

Stronger and/or more synaptic connections made by the repetitive activation of excitatory synapses lead to long-term potentiation (LTP), helping the brain to learn; weaker and/or fewer synaptic connections when there are no excitatory potentials during some time lead to long-term depression (LTD). These changes cause altered neuronal network circuitries that mediate responses and increased flexibility to neural circuits involved in learning and memory [97]. As synaptic connections are mediated by neurotransmitters, it is important to investigate this in more detail.

Neurotransmitters

Neurotransmitters are chemicals that have the role of transmitting signals between neurons in the brain and other parts of the body. They are released from the axon terminals of one neuron and bind to specific receptors on the dendrites or cell body of another neuron, thereby transmitting the signal across the synapse. Different neurotransmitters have different effects on the receiving neuron being excitatory or inhibitory. One example of a neurotransmitter is glutamate, the principal excitatory neurotransmitter in the brain. It acts in the post synaptic terminal generating action potentials which depend on the membrane depolarization produced by glutamate-induced opening of ionotropic glutamate receptors. This opening occurs in three ionotropic receptor families, the *N*-methyl-D-aspartate (NMDA) one of the most abundant glutamate receptor, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionicacid (AMPA) and kainate [98]. Glutamate-induced opening of the NMDA receptor, a very important receptor for controlling synaptic plasticity and memory [56], requires coordinate depolarization of the post-synaptic membrane [97]. Action potentials that release sufficient glutamate to activate alternative postsynaptic glutamate channels such as AMPA-type receptors can depolarize post-synaptic membranes to subsequently allow NMDA-mediated glutamate signalling. This provides a mechanism for LTP at glutamatergic synapses and helps explain why NMDA receptors play a central role in learning. However its activation is only one of many control mechanisms associated with synaptic function; for example, elevations in calcium associated with neurotransmission activate protein kinases and phosphatases that regulate a number of target activities related to LTP and LTD [98].

In addition to glutamate, there is another well-known neurotransmitter, GABA, a primary and key mediator of inhibitory neurotransmission in the central nervous system. At least 2 subtypes of GABA, A and B, have been well defined [99]. GABA-A receptors are found especially in brain and neural tissue [100] and are important for fear conditioning and potentially for regulating processes during fear learning [101]. GABA-A receptors mediate an increase in Cl– conductance across the postsynaptic membrane. While GABA-B

receptors have not been identified as playing a role in the mechanism of action of any anaesthetic agents [102], GABA-A are affected by volatile anaesthetics, such as ethanol [103], propofol [11], and etomidate [72]. GABA-A receptors mediate fast synaptic inhibition by generating transient inhibitory postsynaptic currents. In addition to this, there is also an activation of extra-synaptic GABA-A receptors. These specific GABA-A receptors have different pharmacological and kinetic properties compared with synaptic GABA-A receptors which have been associated to the neurodepressive properties of general anaesthetics [104, 105]. The effects of anaesthetics on tonic inhibition have been characterized in the hippocampus [106], which is crucially involved in learning and memory [107]**.** The binding of certain anaesthetics to GABA-A receptor increases the frequency of the chloride ion channel opening. An increase in Cl– conductance across the postsynaptic membrane causes hyperpolarization and neuronal inhibition. This decreases the probability of action potential firing due to the differences in the frequency of channel opening and/or to the differences in the mean channel opening time, making the cell membrane less likely to depolarize, which contributes to the anaesthetic/ hypnotic state [102].

In addition to ligand-gated ion channels, neurotransmitters generally also interact with metabotropic G-protein coupled receptors (GPCRs). Numerous different GPCRs grouped within synaptic membranes act as a second messenger adapted to a wide range of neurotransmitter inputs. There are at least three different metabotropic dopamine receptors that interact with heterotrimeric G-proteins and generally increase synapse strength, and there are at least two other dopamine receptors that activate other G-proteins that generally decrease synaptic strength [97]. Other known modulators of memory are postsynaptic density proteins (PSD). These proteins have been attributed the role of gathering and organizing neurotransmitter receptors around the synaptic cleft. When an alteration in these is present, they can be responsible for several neurological disorders and disruption of learning and memory [108].

Specific proteins or groups of proteins have been studied throughout the years and used as classical markers for learning, memory, synaptic plasticity, synaptic vesical exocytosis and therefore cognition; examples are described below.

SNARE-SNAP-NSF complex

The process of neurotransmitter release from synaptic vesicles is mediated by the SNARE-SNAP complex, which consists of a set of proteins that regulate vesicle membrane fusion and recycling [109]. Although this process is mediated by many proteins [110], it only makes sense to discuss them as a group since it is the delicate balance between all of them that makes exocytosis possible.

The SNAP-SNARE complex together with the N-ethylmaleimide-sensitive factor (NSF) is a set of proteins that mediates the process of neurotransmitter release at the synapse. The SNARE complex is formed when an action potential arrives at the presynaptic terminal, triggering the influx of calcium ions, which leads to the docking of synaptic vesicles with the presynaptic membrane. The interaction between SNAP-25 and synaptobrevin/VAMP is critical for the fusion of the vesicle membrane with the presynaptic membrane, leading to the release of neurotransmitters into the synaptic cleft. NSF then disassembles the SNARE complex, allowing for recycling of individual SNARE proteins for subsequent rounds of neurotransmitter release [111]. The formation and disassembly of the SNAP-SNARE complex ensure the specificity and efficiency of neurotransmitter release, making it a crucial component of synaptic communication [112].

CAMKII

One of the key effector enzymes in calcium signalling is the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). The holoenzyme CaMKII has four distinct CaMKII isoforms: α β, δ, and γ [113, 114]. Increased intracellular calcium triggers the enzyme's activation, which phosphorylates target proteins involved in several functions, including the mobilization of synaptic vesicles, modulation of ion channels, and LTP [115, 116]. Over the past 20 years, CaMKII has been extensively studied and it all points to it being a critical player in learning, memory, and synaptic plasticity [115, 117].

Synaptophysin

The first synaptic vesicle protein to be cloned and characterized was synaptophysin [118]. It is now known that this protein is a member of a family of four transmembrane domain-containing proteins that also includes synaptogyrin and synaptoporin [119] although synaptophysin is the most abundant one [120]. This protein is frequently employed as a marker for pre-synaptic terminals since it is solely localized into synaptic vesicles. Recent genetic testing on humans and behavioural research on mice have suggested that cognitive deficits result from the loss the synaptophysin gene [121] showing that it may have a significant role in controlling synaptic transmission in neuronal circuits involved in learning and memory[122].

BDNF

The brain-derived neurotrophic factor (BDNF) is a member of a family of neurotrophins that has multiple roles. It regulates both excitatory and inhibitory synaptic transmission [123], activity-dependent plasticity in adults [124] and is essential for the survival and differentiation of neuronal populations during development [125]. BDNF has also been hypothesized to be an integral component of the cellular system supporting memory formation and maintenance by increasing synaptic consolidation that is critical for LTP [126].

PSD-95

One of the most prevalent post synaptic densities proteins is the PSD-95 [127]. The importance of this protein in facilitating synaptic maturation and controlling synaptic strength and plasticity is well-established [128], as it is involved in trafficking, and anchoring synaptic proteins and in stabilizing synaptic changes during LTP [129] [130]. It is usually used as a post synaptic marker [131]. All this points to PSD-95 having also a critical role in memory and cognition.

Conditioning-based methods to study aversion and memory

With emotions triggering behavioural responses, emotions can be activated by stimulus association to study learning. This is the basis of classical conditioning first demonstrated by Ivan Pavlov [132]. Pavlov showed that if a bell was repeatedly played while food was being given to the dogs, they could be conditioned to salivate at the sound of the bell. Salivation was an unconditioned (innate) reaction to the unconditioned stimulus food. Before serving the food, Pavlov rang the bell (a neutral stimulus). After a few repetitions, the dogs began to salivate at the sound of the bell even without being offered food. In this case, salivation was the conditioned reaction to the conditioned stimulus bell. Zebrafish also have the capability of being conditioned.

The most used classical conditioning paradigms to test learning or stimulus valency, such as aversion are the conditioned place preference (CPP) and the conditioned place aversion (CPA). Both paradigms work by pairing a treatment with a distinguishable tactile/visual/auditory/olfactory cue, leading to associative learning. These cues may elicit a pleasant or unpleasant experience (conditioning), and so distinct reactions are reported; in the CPP it is expected that the animal goes towards the conditioned place, for example, while in the CPA it is expected that the animal avoids that place.

Although several different designs and apparatuses are used, the basic design consists of a two-compartment chamber with being different characteristics (e.g., white vs. black walls and floor, a dark vs an illuminated side, different water depth, and different floor patterns) connected by a doorway. The gate between the compartments can be opened to allow an animal to pass freely between them. Usually, the main measurement is the latency to enter in the conditioned compartment, although time spent on each compartment can also indicate conditioning learning or aversion.

Some of these tests require a lengthy behavioural protocol that must be repeated multiples times to train the animals. When a quick learning is required, training the animal for several days may be a limitation, as it is hard to identify the moment when the animal learns [133]. Nevertheless, there is a simple learning protocol based on fear conditioning and passive inhibitory avoidance of a threatful stimulus that needs to be learned as quickly as possible - One Trial Inhibitory avoidance task [134]**.** This behavioural test was first developed for rodents [135]. Rats were subjected to a single aversive shock into a darkened compartment (their preferred compartment). To study whether the training was retained 24 or 48 hours later, the rats were placed in the lighted compartment and their latency to enter the former shock chamber is measured; longer latencies are seen as signs of better memory. Although inhibitory avoidance training only involves one trial, the complex brain processes that underlie learning and memory are still present [136]. This test was adapted to zebrafish using a black and white arena [134] to study the influence of drugs on memory. Zebrafish prefer the black compartment; thus, the shock was given in this side.

Regarding aversion, it can be measured by associating the treatment we want to study to a preferred place [9, 134]. If the animal does not maintain the normal preference, it indicates that the animal is avoiding a previous preferred place, thus, revealing aversion to the treatment.

Aims

Zebrafish is commonly used in research, enduring procedures that require anaesthesia. As anaesthesia is routinely used, it is critical to refine and study its potential side effects, such as aversion and memory. Aversion can interfere directly with animal welfare inducing distress in the animals, thus, potentially altering research. Also, memory is a cascade of biochemical processes in which its complexity can be affected by external events such as anaesthesia [39]. As there is little information about zebrafish, studying anaesthetic effects on memory using a behavioural approach complemented with molecular techniques might enlighten some aspects about the basis of the mechanisms behind anaesthesia and its effect on memory. This knowledge could be used as the basis for a translational knowledge that will benefit both humans and animal models as anaesthesia and memory mechanisms are conserved across species [137]. Thus, the general aim of this work is to study some often-disregarded side-effects (aversion and memory) of the standard anaesthetic MS222, and the combination propofol/ lidocaine proposed by our group to be used in adult zebrafish. The specific aims step by step are:

- **Study the potential aversive profile of different anaesthetics (MS222, propofol/ lidocaine, etomidate, clove oil).**
- **Set up and validate the apparatus to study learning/ memory in zebrafish, using the one-trial inhibitory avoidance.**
- **Study the potential effects of promising anaesthetic protocols on memory.**
- **Study the synaptosome proteome involved on conditioning learning.**
- **Study the impact of promising anaesthetic protocols on the synaptosome proteome.**

Chapter 1 - Do different anaesthetic protocols produce aversion and modulate cortisol levels in adult zebrafish?

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Introduction

According to the latest European Commission (EU) report on the statistics on the use of animals for scientific purposes in the EU [138], fish are the second most used animal group in research (13%); with zebrafish representing 40% of these. The refining of procedures is essential for responsible high-quality research. As several procedures involve stress and/or pain, the use of anaesthesia is often required. Most studies only evaluate the efficacy of anaesthetic protocols in terms of their capacity to induce anaesthesia that is adequate for the procedure, providing immobility and/or analgesia, and preventing physical harm and distress. However, the potential aversive effect of anaesthesia is poorly studied, raising welfare concerns. In fact, only two studies [7, 10] were performed to study this issue in zebrafish, and more data are needed to evaluate which protocol fully respects zebrafish welfare.

MS222 or tricaine has been shown to be aversive to adult zebrafish, and this aversion was independent of its acidic profile, as animals showed signs of aversion or distress regardless of the anaesthetic solution being buffered or not [7, 10, 55, 139]. Nevertheless, MS222 is the standard and most used anaesthetic in zebrafish research [140], probably due to its traditional use in larger fish species, as it is the only anaesthetic approved by the Food and Drug Administration agency as safe to use in the fish industry. Considering the risk of causing aversion, the development of alternative anaesthetic protocols to provide better animal welfare and produce more reliable scientific data is needed. Previous studies suggested the combination of propofol with lidocaine, clove oil, and etomidate as being promising alternatives [7, 9, 10].

Propofol, a short-acting sedative-hypnotic, consistently induces analgesia in zebrafish but only at high concentrations [65], according to recent studies [57], which may raise the danger of overdosing. Other studies have shown that immersing zebrafish in a water bath containing a high concentration of the local anaesthetic lidocaine had analgesic and/or anaesthetic effects [61, 64], but this also increased zebrafish mortality [61]. However, when combining propofol with lidocaine, the dose of each individual anaesthetic can be decreased [1, 65, 141], inducing a balanced and safer anaesthesia. Another anaesthetic used is clove oil, which is highly lipophilic and rapidly absorbed through the skin and gills, entering the bloodstream, crossing the blood–brain barrier, and inducing loss of consciousness. Clove oil has been demonstrated to minimise cortisol response compared with MS222 [142, 143]. This agent is widely available, has a low cost, and has a wide range of efficacy (i.e., from 2 to 5 µg/mL for sedation to 60 to 100 µg/mL for immersion anaesthesia) [56], but it needs to be solubilised in an organic solvent such as ethanol prior to anaesthesia, which increases the workload while introducing one more chemical compound [69, 70]. Another potential

downside is that clove oil contains low levels of methyleugenol, which is considered carcinogenic [144]. Contrarily, etomidate is a safe nonbarbiturate hypnotic agent that has been described to block cortisol [72, 145], the same effect its derivative metomidate has in fish [142, 146]. Etomidate does not provide analgesia, but it is a relatively fast-acting anaesthetic agent [1].

In order to improve anaesthesia for adult zebrafish, the study's primary goal was to identify the least aversive anaesthetic protocol when testing the MS222, propofol/lidocaine, clove oil, and etomidate anaesthetics. The conditioned place aversion test (CPA) and cortisol level quantification were used to assess zebrafish behavioural aversion and physiological stress.

Materials and Methods

Ethics Statement

All procedures were carried out under personal and project licenses approved by the National Competent Authority for animal research (Direção-Geral de Alimentação e Veterinária, Lisbon, Portugal) (approval number: 014703) and by the Animal Welfare and Ethics Review Body of the Institute for Research and Innovation in Health (i3S). The European Directive 2010/63/EU on the protection of animals used for scientific reasons, as well as its transposition into Portuguese law ("Decreto Lei" 113/2013), were followed in all experimental procedures.

Animals and Housing

Sample-size calculation was performed in G*Power 3.1 (University of Düsseldorf, Düsseldorf, Germany) while assuming a type II error probability of $\alpha = 0.05$, a power of 0.85, and an effect size of 0.988 based on previous data [9]. Adult (7–16 months old) mixed-sex AB zebrafish (N = 177; 81 for CPA + 96 for cortisol) bred in the Animal Facility of i3S were used. They were kept in groups of eight fish in 3.5 L tanks on a 14 h:10 h light:dark cycle in a recirculating water system connected to a central unit of water purification under controlled conditions (27.0 ± 0.5 °C, pH of 7.0 ± 0.5, and conductivity of 700–715 S). Fish were fed thrice daily with a commercial diet (Zebrafeed 400–600 µm, Sparos, Olho, Portugal). The same water and light:dark cycle conditions were maintained in the conditioned place aversion apparatus where the animals were housed during the experiment.

Anaesthetic Solutions Tested

For this study, we used 69 mixed-sex zebrafish (57% male to 43% female) randomly divided into six groups: HCl (animals subjected to water with a pH of 3 induced by hydrochloric acid; n = 11), MS222 (Sigma-Aldrich, Burlington, MA, USA) (n = 12), Propofol/Lidocaine (P/L) (propofol was Lipuro 2%, B. Braun Melsungen AG, Germany, and 2%, Braun, Queluz de Baixo, Barcarena, Portugal) (n = 12), Clove Oil (Merck, Kenilworth, NJ, USA) (n = 12), Etomidate (Lipuro 2 mg/mL, B. Braun, Queluz de Baixo, Barcarena, Portugal) (n = 10), and Vehicle (Intralipid, Sigma-Aldrich, Burlington, MA, USA) (n = 12); the concentrations of the compounds used in these groups are displayed in Table 1. The pH of all solutions was around 7 (6.97 ± 0.11) (Table 1.1). The animals from the anaesthetic groups were exposed to equipotent concentrations to induce loss of equilibrium that were established prior to the study [9]. All anaesthetic solutions were freshly prepared except the MS222 and clove oil. The MS222 test solution was prepared from a buffered (pH = 7) stock solution (10 g/L) and the clove oil test solution from a stock solution of 10% clove oil and 90% ethanol.

Hydrochloric acid at a pH of 3 is aversive to zebrafish [7], thus a HCl group was used as a positive control to validate the behavioural methodology used to study aversion. As propofol and etomidate have a white colour, the fish could react to visual cues and not to the actual exposure to the anaesthetics. To rule out this influence, a group exposed only to the common vehicle of these compounds (Intralipid) was added as a positive control for the white colour—Vehicle group. For the P/L and the Etomidate groups, during the Post-exposure phase, the Intralipid vehicle was added to the water at the same concentration as was present in the anaesthetics, thus mimicking their colours. The Vehicle group had the same concentration of Intralipid as the propofol in the P/L group. The other testing solutions were transparent (see annex 1).

	Concentration	pH
MS222	150 mg/L	7.05
Etomidate	2 mg/L	6.97
Vehicle for etomidate	60 mg/L Intralipid	6.86
Clove oil	45 mg/L	7.15
Propofol/lidocaine	5 mg/L propofol + 150 mg/L lidocaine	7.05
Vehicle for propofol/lidocaine	5 mg/L Intralipid	6.93
Vehicle	5 mg/L Intralipid	6.93
HCl	4.67 mg/L	3.00
System water		6.94

Table 1.1. The pH of the solutions and the system water used in the study as measured by a pH probe (Inolab, level 1, Xylem Analytics Germany Sales GmbH & Co. KG, Oberbayern, Germany). The solution temperature was 26 ± 1 °C.

Conditioned Place Avoidance Test

To test behavioural aversion, we used a methodology adapted from Wong et al. [10] and Ferreira [9]. This experiment was carried out in an apparatus that had two opaque black aquariums connected by a tube so that the fish could travel between them; each tank had a small transparent window (Figure 1.1).

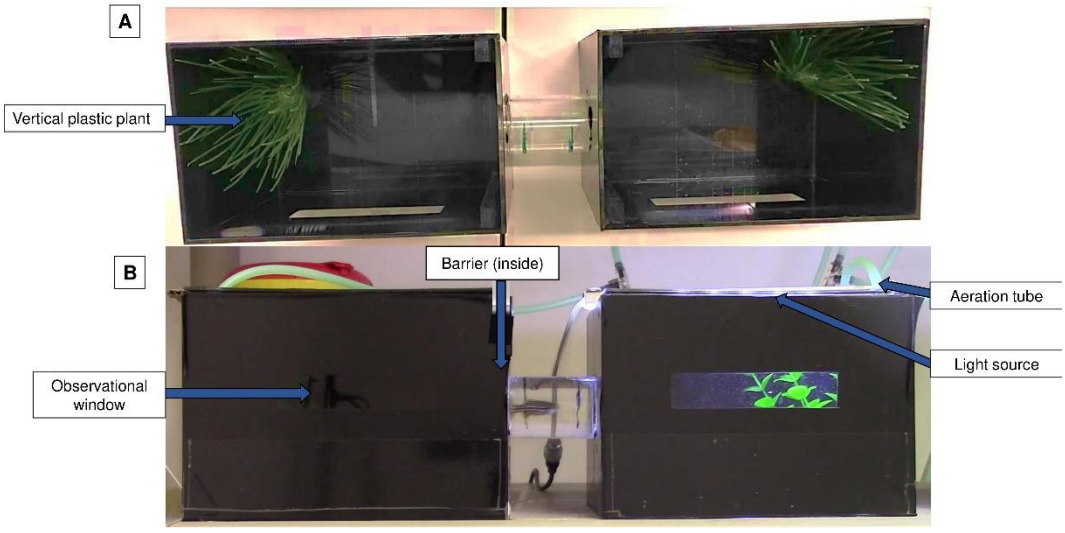

Figure 1.1: Conditioned place aversion apparatus view from above (**A**) and from the side (**B**). Tanks were individually lighted and only one tank had the lights on at a certain time. A transparent tube linked the tanks for the animals to pass; a plant and aeration were provided on each side to increase animal welfare.

Each aquarium had a lid equipped with an LED light source, but only one of the LED lights was lit at a given time. Vertical plastic plants (from a pet store) were added as an environmental enrichment to minimise the possible negative impact of isolation [147]. Plants were always placed on the opposite wall of the tube on both sides of the apparatus. Aeration was additionally provided and was positioned near the plant. The conditioned place avoidance paradigm involved associating an aversive experience with a setting that was previously viewed as neutral or favourable, leading to the avoidance of the neutral or preferred location [146]. The degree of avoidance was proportionated to the time spent on each side of the apparatus and the latency to enter an environment that was previously perceived as neutral or positive [7] (Figure 1.2). The animal remained inside this apparatus for the entire conditioned place avoidance test, which had a maximum duration of 13 days.

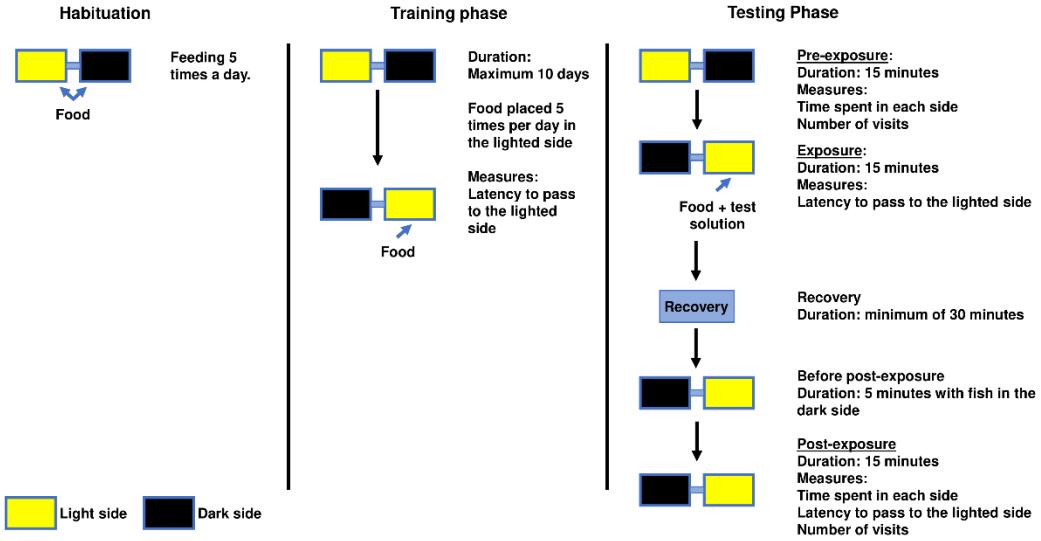

Figure 1.2: Conditioned place aversion behaviour test scheme with measurements taken in each phase. The habituation phase lasted for 48 h followed by a training phase in which the training baseline was established; training was completed when the criteria for fully trained animals was reached (see the Materials and Methods section). Afterwards, the testing phase was divided into pre-exposure and post-exposure performed on the same day. Recovery represents the recovery of the anaesthesia or other testing solution when the animal was placed in clean water. Which tank was lighted at each given time and where the food and testing solutions were introduced are also depicted.

Habituation

This first phase, which lasted 48 h, involved placing each animal separately in the apparatus for acclimatisation. Animals were fed five times a day on the side where they were found during this phase. The animals were only routinely observed for health at this point without any testing being performed.

Training Phase

Animals were moved to the lighted side of the apparatus during this phase before being fed by being gently pushed with a net in the direction of the tube; animals that were already on that side were also subjected to contact with the net without being exposed to the air. The placement of a transparent partition ensured that the animals remained on the lit side and were unable to cross over to the darkened side. Then the sides' lighting was switched, food was added to the newly lit side, the partition was lifted, and the animals were free to move around and eat. The animals' innate preferences for this environment were reinforced by feeding them on the lit side. Additionally, the animals were trained to pass through the tube after the removal of the divider and the light switch, which served as cues to indicate the presence of food on the other side. Five trials per day were used to measure the latency to enter the lit side during this phase following the light switch and partition removal. This phase lasted for up to 10 days or until the subjects met the criteria for being fully trained, which was when the subjects entered the lit side at least three times in a period of one minute or less and never took more than two minutes in the remaining trials on any given day for three consecutive days. The five latencies to enter the lighted side in the last day of training were averaged and designated as the training baseline, which was later compared with the latency of the testing phase.

Testing Phase

Pre-exposure: a total of 24 h after being fully trained, animals were video-recorded for 15 min without any interference and the time spent in each side was noted.

Exposure: immediately after pre-exposure, the transparent barrier was placed when the fish was on the lighted side while the test solution and the food were introduced on the opposite side. At this point, the light conditions were switched between sides and the barrier was lifted. Fish were expected to quickly enter the lighted side, similarly to the training baseline. This trial ended when an animal spent 3 consecutive minutes on the side with the anaesthetic/test solution or when 15 min elapsed irrespective of the animal's location. Three minutes was considered a safe amount of time to ensure that the animal was unconscious [141] and there was no risk of overdose; this was established because monitoring the fish was difficult due to the apparatus being opaque and having only a small window. After this trial, fish recovered in a tank with clean water from the system for at least 30 min; after this period, all animals exhibited normal swimming and behaviour. Animals that did not enter into contact with the test solution were not further evaluated. In this phase, only latency to enter the lighted side was recorded.

Post-exposure: following recovery, the animal was introduced again into the darkened side of the experimental apparatus with the barrier placed on this side for five min to prevent the fish from passing to the lighted side. Following the 5 min introduction period, the barrier was lifted and the animal was allowed to explore the entire apparatus for 15 min to verify if there was any evidence that the fish was conditioned to avoid the side on which it was previously exposed to the test solution. In this trial, no anaesthetic or HCl solution was added to the water. However, the P/L and etomidate groups were also exposed to the vehicle in this phase so that the visual cue (white colour) was present without the active compound of the anaesthetics (see annex 1). To evaluate the aversion caused by each anaesthetic, data on the length of time spent in each side, the number of visits, and the latency to enter the lighted side were recorded. The trial was over after 15 min regardless of whether the fish entered the lit side. In this case, a value of 900 s (maximum latency) was attributed to these animals regarding the latency to enter in the lighted side. All videos were recorded with a camera (Legria HF R606, Canon, Ōta, Japan) facing the tunnel and were later analysed with the use of Ethowatcher (Universidade Federal de Santa Catarina, Santa Catarina, Brazil) [147], a behavioural-event-logging software.

To ensure that there was no significant diffusion of the anaesthetic to the opposite side of the apparatus, a trial using food colouring and without any fish was conducted prior to the CPA. The minimal diffusion of this substance to the opposite side after 30 min (more than the trial's duration) suggested that little to no testing solution—if any—entered the apparatus's darkened side during the testing phase.

Cortisol Determination

Trunk cortisol levels have been assessed previously as an indication of stress response in smaller adult fishes such as zebrafish [148, 149]. Another batch of zebrafish were euthanised by rapid cooling followed by decapitation 5 or 15 min after loss of equilibrium in response to the anaesthesia exposure (n = 12). If they were females, the eggs were gently removed by pressing their abdomen [150] and then each trunk was weighed and placed in a 15 mL centrifuge tube at −20 °C containing 5 mL of ice-cold phosphate-buffered saline (PBS, pH 7.4). Trunk samples were processed for cortisol measurement as previously described [151]. Samples were rapidly thawed on ice and homogenised twice in 500 µL of PBS using the automatic lyser TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 90 s. Cortisol was extracted into 500 µL diethyl ether and vortexed for 30 s. Samples were further centrifuged at 5000 rpm (Heraeus Biofuge Pico, Hanau, Germany) for 10 min and frozen at −20 °C to collect the organic layer. The extraction was repeated twice and the final tubes containing the diethyl ether extract were dried at 45 °C under reduced pressure in a

Labconco centrifugal evaporator (model: Centrivap 78120-00 D). The dried extracts were portioned with 500 µL of PBS and the same amount of hexane to prevent interference from the precipitated lipids. Samples were then vortexed and centrifuged as before. The aqueous phase was stored at −20 °C until analysis. For the analysis of cortisol levels, 50 µL of the reconstituted samples was used and assayed at 405 nm with a correction at 490 nm on a microplate spectrophotometer (PowerWave XS2, Bio-Tek Instruments, Winooski, VT, USA) by following the ELISA kit instructions (Salimetrics assay #1-3002; Salimetrics, State College, PA, USA). Values are presented in ng per gram of the animal body weight.

Data Analysis

Response variables (latency to enter the lighted side, time spent on the lighted side, cortisol levels, number of entries on the lighted side, and probability of entering the lighted side at least once) were tested as functions of the studied fixed and random effects, as detailed below. Different modelling approaches were used for each responsible variable to ensure a normal distribution of residuals and homoscedasticity. For all fitted models, least-square means were compared while considering a 95% confidence interval with Tukey–Kramer corrections for multiple pairwise comparisons. All the analyses were performed using SAS University Edition® (SAS Institute Inc., Cary, NC, USA).

For analyzing latency to enter the lighted side, the GLIMMIX procedure was used with a log-normal response distribution for testing latency to reach the lighted side (response variable) as a function of the fixed-effect treatment group (six-level categorical variable with levels of HCL, Etomidate, Clove Oil, P/L, Vehicle, and MS222), fixed effect of phase (three-level categorical variable with levels of the training baseline, exposure, and post-exposure), and the interaction between these effects while considering fish as random effect.

For the analysis of time spent in the lighted area, an index was calculated by dividing the total time spent by the fish on the lighted side by the summation of the time spent on both sides; we excluded the time spent by the fish in the communicating tube because there was substantial variation among individual fishes in the total time spent in the tube. In this way, the calculated index was an indication of the proportion of time spent in the lighted area relative to the total time that the fish was in one of the areas. To achieve a normal distribution of model residuals and homoscedasticity, a square-root data transformation was performed on the calculated lighted-side index. The MIXED procedure was used for testing the square-root-transformed lighted-side index (response variable) as a function of the fixed-effect treatment group (six-level categorical variable with levels of HCL, Etomidate, Clove Oil, P/L, Vehicle, and MS222), fixed effect of phase (two-level categorical variable with levels of pre-

exposure and post-exposure), and the interaction between these effects while considering fish as a random effect.

Two different approaches were used to evaluate fish entrance into the lighted side. First, the probability of fish entering the lighted side at least once was modelled via logistic regression by using the LOGISTIC procedure while considering the fixed-effect treatment group (six-level categorical variable with levels of HCL, Etomidate, Clove Oil, P/L, Vehicle, and MS222). Second, the number of entries in the lighted side was log-transformed to achieve a normal distribution of model residuals and homoscedasticity and regressed as a function of the fixed-effect treatment group (six-level categorical variable with levels of HCL, Etomidate, Clove Oil, P/L, Vehicle, and MS222) using the GLM procedure. To allow for the log-transformation, a constant 0.1 value was added to all data points to eliminate zero values. The number of days taken for the fish to learn to enter the lighted side was tested as a covariate in the model but removed due to a lack of significance.

For the cortisol analysis, the GLM procedure was used to test the cortisol level (response variable) as a function of the fixed-effect treatment group (six-level categorical variable as previously mentioned), fixed effect of sex (two-level categorical variable: female and male), fixed effect of time of sample collection after equilibrium loss (two-level categorical variable: 5 or 15 min), and the interaction between treatment group and sex.

Results

Animals Removed from the Analysis

Two animals from the P/L group and one animal from the etomidate group were not considered for the conditioned place avoidance analysis because they did not enter the lighted side during the exposure phase and thus did not have any contact with the conditioning agent. In addition, a total of nine animals were excluded from the cortisol analysis (two animals exposed for 5 min and four animals exposed for 15 min to etomidate; and one animal exposed for 5 min and two animals exposed for 15 min to P/L) because the cortisol levels in the biological samples from these animals were below the detection limit of the method used.

Conditioned Place Avoidance Test

All Animals Took Longer to Go to the Lighted Side after Conditioning, Especially HCl Animals

Figure 1.3 illustrates the latency to enter the lighted side. This variable was significantly ($p \leq 0.0002$) affected by the treatment group, phase, and the interaction between these two variables. Latency to enter the lighted side during the training baseline was considered a control; no differences were observed between groups ($p > 0.05$) for this phase. Regarding the exposure phase, MS222 animals were faster to enter the lighted side when compared with the latency of the HCL and the Etomidate groups ($p \leq 0.0194$). Regarding the post-exposure phase, animals from the MS222, Clove Oil, P/L, and Vehicle groups had a lower latency to enter the lighted side than the HCL group ($p \leq 0.0060$). Moreover, animals treated with etomidate took longer to enter the lighted side than the MS222 group ($p = 0.0499$). All groups took less time to enter the lighted side during the training baseline when compared with the exposure ($p < 0.0001$) or post-exposure ($p < 0.0001$) phases. Animals in the MS222, Vehicle, and HCl groups also showed an increased latency to enter in the lighted side in the post-exposure phase compared with the exposure phase ($p = 0.0090$, $p = 0.0400$, and $p < 0.0001$, respectively).

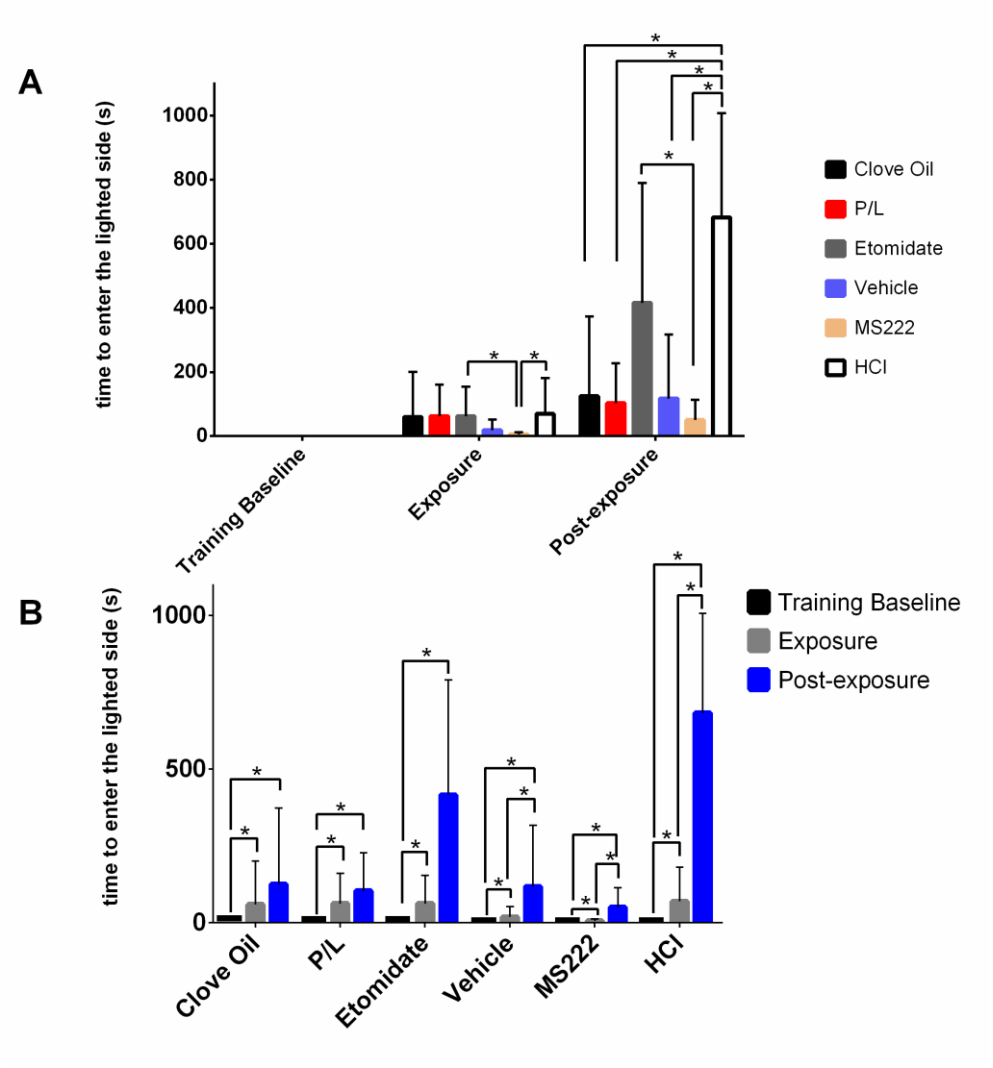

Figure 1.3: Observed median latency to enter the lighted side of the apparatus and their respective interquartile ranges on the last day of training (Training Baseline) in the exposure phase and in the post-exposure phase for each treatment group. Clove Oil group: animals subjected to 45 mg/L of clove oil (n = 12); P/L group: animals subjected to 5 mg/L of propofol combined with 150 mg/L of lidocaine (n = 10); Etomidate group: animals subjected to 2 mg/L of etomidate (n = 9); Vehicle group: animals subjected to 5 mg/L of intralipid (n = 12); MS222 group: animals subjected to 150 mg/L of MS222 (n = 12); HCl group: animals subjected to pH 3 water with hydrochloride acid (n = 11). Animals that did not enter into the lighted side had an attributed latency of 900 s. (**A**) Illustration of the comparisons between groups within each testing phase; * $p < 0.019$. (**B**) Illustration of the differences between each phase for each experimental group; * $p < 0.040$. The analysis was performed using the GLIMMIX procedure with a log-normal response distribution; asterisks indicate significant differences between least-square means. The training baseline latencies were so low that they are not visible in panel A; the corresponding bars were made visible in panel B to facilitate the figure legibility (Clove Oil: 7.5 (7.3) s, P/L: 6.0 (9.8) s, Etomidate: 9.0 (2.3) s, Vehicle: 8.5 (10.8) s, MS222: 9.0 (7.3) s, HCl: 6.0 (7.0) s).

Positive Control Group Animals Spent Less Time on the Lighted Side and Did Not Differ from the Etomidate Group

Figure 1.4 illustrates the time spent on the lighted side. This variable was significantly affected by the treatment group (p = 0.0154) and the interaction between the treatment group and phase (p = 0.0003). Regarding the pre-exposure phase, no differences between groups were observed for time spent in the lighted side. In the post-exposure phase, fish from the MS222, Clove Oil, P/L, and Vehicle groups spent more time on the lighted side than the HCL group ($p \leq 0.0022$). In addition, fish from the HCl group spent less time on the lighted side during the post-exposure phase compared with the pre-exposure phase (p = 0.0164). The other groups spent similar times in the lighted side before and after conditioning.

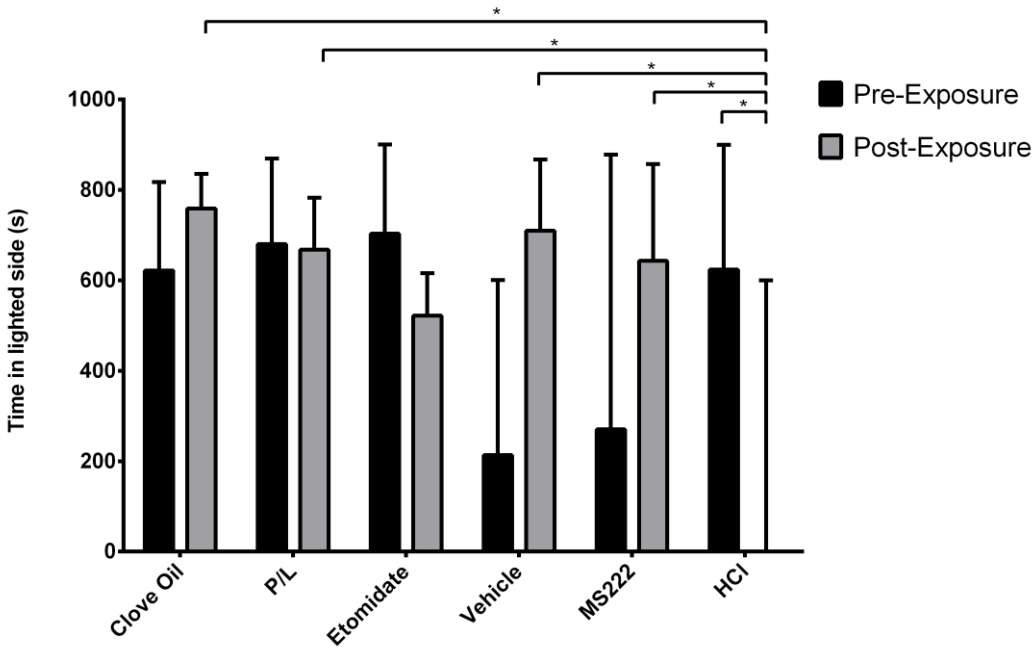

Figure 1.4: Observed median time spent on the lighted side of the apparatus and their respective interquartile ranges in each treatment group during the pre-exposure and post-exposure phases. Clove Oil group: animals subjected to 45 mg/L of clove oil (n = 12); P/L group: animals subjected to 5 mg/L of propofol combined with 150 mg/L of lidocaine (n = 10); Etomidate group: animals subjected to 2 mg/L of etomidate (n = 9); Vehicle group: animals subjected to 5 mg/L of intralipid (n = 12); MS222 group: animals subjected to 150 mg/L of MS222 (n = 12); HCl group: animals subjected to pH 3 water with hydrochloride acid (n = 11). * $p \leq 0.016$ using the MIXED procedure for testing the square-root-transformed lighted-side index (time spent on the lighted side/total time spent in both sides); asterisks indicate significant differences between least-square means of groups and phases.

Positive Control Group Animals Entered the Lighted Side Less Often

Regarding the post-exposure phase, the number of entries into the lighted side was significantly affected by the treatment group ($p < 0.0001$). Here, fish from the MS222, Clove Oil, P/L, and Vehicle groups entered more often into the lighted side than the HCL group ($p \leq 0.0012$) (see annex 2). The probability of an animal to enter the lighted side at least once was not affected by the treatment group. Nevertheless, all animals from the MS222, Vehicle, and P/L groups entered the lighted side after conditioning, while one animal from the Clove Oil group, three animals from the Etomidate group, and more than half the animals from the HCl group did not enter the lighted side (7/11).

Etomidate Animals Showed Lower Levels of Cortisol

For cortisol samples, sex was not found to have a significant effect. Time of collection after equilibrium loss ($p = 0.0001$) and interaction between this variable and treatment group ($p = 0.0182$) were significant, but no differences were found between treatment groups in samples collected 5 min after equilibrium loss (Figure 1.5). Regarding the samples collected 5 min after equilibrium loss, animals exposed to etomidate had significantly lower cortisol levels compared with the other groups ($p \leq 0.0001$, $p = 0.0018$, and $p = 0.0025$ for the MS222, Clove Oil, and P/L groups, respectively) (Figure 1.5). The MS222 group was the only one with a significantly higher level of cortisol at 15 min when compared with the cortisol levels at 5 min ($p = 0.0034$) (Figure 1.5).

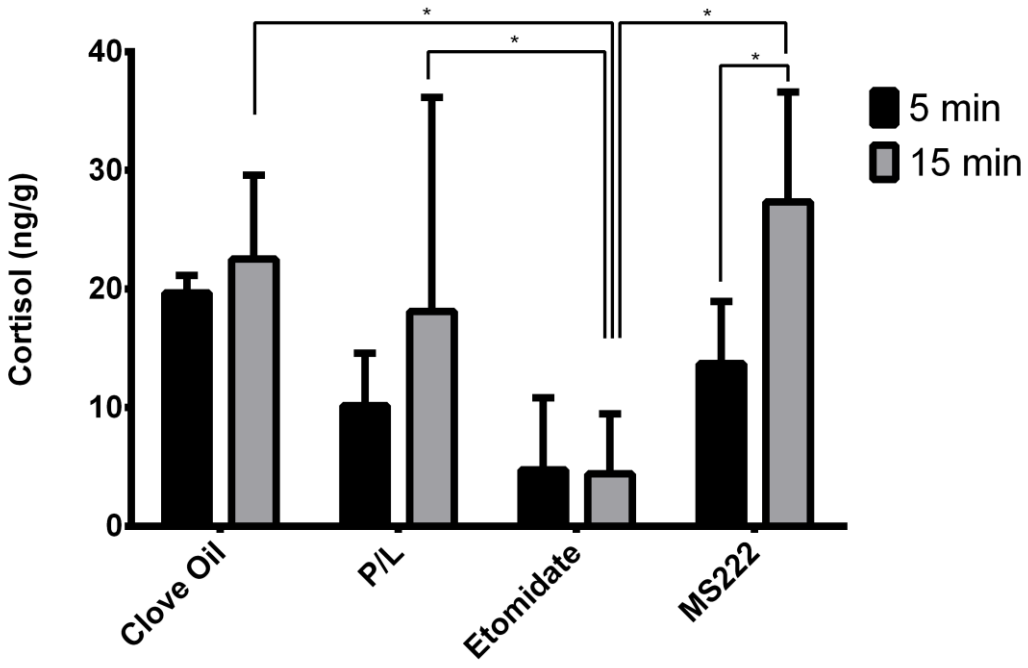

Figure 1.5: Observed medians of trunk cortisol levels 5 min or 15 min after zebrafish had lost their equilibrium and their interquartile ranges. Clove Oil group: animals subjected to 45 mg/L of clove oil (n = 12); P/L group: animals subjected to 5 mg/L of propofol combined with 150 mg/L of lidocaine (n = 11); Etomidate group: animals subjected to 2 mg/L of etomidate (n = 10); MS222 group: animals subjected to 150 mg/L of MS222 (n = 12). * $p \leq 0.003$ significant differences between least-square means of groups and phases using the GLM procedure.

Discussion

Anaesthetic efficacy and potential side effects should be considered when selecting an anaesthetic protocol to ensure both animal welfare compliance and provision of scientifically sound data. One important aspect to consider is whether an animal perceives a drug as aversive, since this would alter the animal's behaviour and physiology, potentially inducing distress and reducing welfare. Unfortunately, the most used anaesthetic, MS222, has been described as aversive to adult zebrafish [7, 10], which motivated our search for better alternatives. The anaesthetic protocols tested in this study (MS222, propofol/lidocaine, clove oil, and etomidate) were not innocuous, as the animals subjected to these behaved differently before and after anaesthesia exposure. However, the MS222, P/L, and Clove Oil groups had a different behavioural profile compared with the positive control for aversion (HCl), contrary to etomidate. Regarding cortisol, all anaesthetic treatments except etomidate presented similar levels between them. The pH of the solution could contribute to the aversion levels, as seen in the HCl group (pH 3) where aversion was clearly present. Thus, we tested the remaining solutions and the pH influence was ruled out because they all had a pH around 7 (Table 1).

Contrary to what was found by Readman et al. [7], in the present study, animals exposed to etomidate showed some signs of aversion, whereas there was no clear aversion profile for MS222. These differences can probably be explained by the use of a different paradigm; in the study reported by Readman et al. [7], the animals moved freely between two sides, one containing only water and the other containing an anaesthetic solution of 50% of the effective anaesthetic concentration recommended [140]. In addition, the period of habituation to the apparatus was only 150 s, while in our experiment it was 48 h. Thus, our paradigm involved conditioned learning, while Readman et al. used a preference test, and the concentration we used was higher to mimic an experimental situation in which the animals had to be anaesthetised and may have felt aversion.

Our previous work [9] attempted to replicate the Wong et al. [10] experiment, but contrary to their results, animals treated with MS222 did not present a clear aversion. Animals were tested in pairs in the Wong et al. study, so the behaviour of one of the fish may have influenced the other, possibly altering the test results due to shoaling or territorial behaviours. Thus, in the present study, the animals were tested individually. During this period of short social isolation (13 days), the environment was enriched with a plastic plant [152]. In the present study, the results from clove oil-treated animals presented were similar to those reported by Wong et al., although they used a higher concentration. However, the MS222-treated animals in Wong et al. showed aversion by spending more time on the darkened side and avoiding the lighted side after conditioning, whereas our results showed

no difference regarding this measure. In our study, the only group that significantly avoided the lighted side was the HCl group, which worked as the positive control for aversion. This control group showed that other dependent variables were relevant to measure aversion: the number of entries into the lighted side and the latency to enter into this side after conditioning; i.e., animals spent less time, exhibited fewer entries, and took more time to enter into the lighted side after being exposed to a pH of 3. Thus, these were the behavioural benchmarks presented by the animals that showed aversion to the anaesthetic/treatment.

Our previous studies [9] showed that animals subjected to propofol/lidocaine took more time to enter the compartment where the anaesthetics were presented than in the training baseline. We suspect that this may have been due to the white colour of propofol. In the present study, etomidate also had a white colour. To rule out the influence of the colour as a visual cue, a group of animals was exposed to Intralipid, the white vehicle of propofol and etomidate (see annex 1). When analysing the results, fish from the Vehicle group took longer to enter the lighted side after being exposed compared with the exposure phase. The P/L and Etomidate groups showed no differences in latency when comparing the exposure phase with the post-exposure phase. All these groups had Intralipid in the water during the post-exposure phase. These results pointed to the colour not being an aversive cue (lack of differences in latency between the exposure and post-exposure phases in P/L and Etomidate), but the re-exposure to the Intralipid alone may have been an aversive factor, which only occurred in the Vehicle group. This finding ruled out the possibility of visual cues skewing the results in the anaesthetic groups during the post-exposure phase. Nevertheless, in the exposure phase, all animals took more time to pass into the lighted side containing the treatment than in the training baseline, probably because they sensed the chemicals in the tube and/or, in the case of the P/L, Vehicle, and Etomidate groups, they observed a visual white cue. The strong odour from clove oil and the acidic properties and odour or taste of HCl [153] may also have been detected in the tube, thus increasing the latency to pass into the treated side during exposure. While the HCl animals took much more time to pass to the lighted side during the post-exposure phase than during the exposure phase, indicating a strong aversion, the same significant difference in the MS222 group may have been caused by the particularly low latencies presented during exposure.

Nevertheless, no anaesthetic protocol seemed to be completely innocuous, as all groups took more time to pass to the lighted side after being exposed. However, in the post-exposure phase, except for the Etomidate group, all the other groups exhibited significantly lower latencies to enter the lighted side compared with the ones from the positive control group. Thus, after a brief initial hesitation, the animals exposed to the anaesthetics (except to etomidate) behaved as if they found the conditioned side safe during the post-exposure phase. The HCl and Etomidate groups were found to spend less time and entered less often

into the lighted side after conditioning, indicating an aversive profile for etomidate because it showed the same results as the positive control group.

It has been described that MS222 induced a long-term but not a short-term memory impairment in Medaka fish (*Oryzias latipes*) and rats [154, 155], and it also induced working memory and cognitive flexibility impairment in adult zebrafish [6]; whereas etomidate induced amnesia [156], although no studies using zebrafish are known. Both lidocaine and propofol induced post-anaesthetic amnesia in other animal models, especially by impairing the consolidation of information retrieved immediately before or after the anaesthetic episode [157-160]. Contrary, clove oil has not been described to impair memory, and there are some studies related to memory improvement when animals were subjected to an amnesic [161]. Except for clove oil, the anaesthetics tested have been described to produce amnesia at some instance in different animal models. Thus, the lack of differences between the pre- and post-exposure phases regarding time spent and number of visits to the conditioning side could have been because the animals forgot the anaesthetic exposure. However, if the animals suffered from amnesia, they would have had similar latencies to enter into the lighted side before and after the conditioning, which did not occur. Nevertheless, in the case of the P/L group, the increase in latency can be explained by the presence of a visual cue on the lighted side during the post-exposure phase and not by this combination causing aversion. Therefore, the presence of an amnesiac effect could not be completely excluded. Although a visual cue was also present in the post-exposure phase for the Etomidate group, there were other dependent variables that showed an aversion degree similar to that of HCl.

Regarding cortisol, levels were not different between groups except for the Etomidate group, which presented lower cortisol levels at 15 min. This was expected because etomidate inhibits the pathway that leads to cortisol synthesis [68, 146]; this can be problematic in several areas of research, so the anaesthetic selection must be carefully considered. Only the MS222-treated animals had higher cortisol levels at 15 min than at 5 min. Indeed, cortisol has been described to peak between 15 to 30 min after a stressful event in fish [160], and it is possible that the MS222-treated animals had a quicker cortisol release than the other groups. The values presented here at 15 min (except for the Etomidate group, as explained) were higher than what we found as baseline cortisol levels in animals euthanised with rapid cooling (mean ± SD of 11.52 ± 5.49 ng/g) [162]. Thus, anaesthesia induction may be a stressful event, as also shown by the behavioural data in which the anaesthetic exposure was not innocuous. We do not know if the stress was related to the chemical compounds per se or if it was behaviourally mediated. The latter explanation seems more likely because the chemical compositions of these anaesthetics

are very different, and the animals presented high levels of cortisol in all the groups (except the Etomidate group) when compared with the baseline reference.

Conclusions

In sum, the stress of anaesthesia induction as measured according to cortisol levels was similar between groups and higher than the baseline reference except for the Etomidate group. This group showed a behavioural profile similar to the positive control group (HCl) in the CPA test, thus presenting some degree of aversion; this did not contradict the low level of cortisol detected, as this result may have been due to the pharmacological effect of etomidate inhibiting cortisol synthesis. Nevertheless, it cannot be assured that any of these anaesthetics are innocuous and do not cause aversion in adult zebrafish, as the latencies to enter the lighted side were altered after exposure to these agents. It is possible that the loss of control of the body during anaesthesia induction could be enough to elicit aversion and a stress response. Except for Etomidate group, the other variables measured to detect aversion did not change after conditioning. Therefore, future studies using different behavioural paradigms that assess brain activity will be important to clarify anaesthesia aversion. In addition, it will also be important to clarify the post-anaesthetic amnesia using these anaesthetic protocols.

In conclusion, according to our results, we cannot recommend the use of the etomidate protocol to avoid aversive responses, but all the other anaesthetic protocols (propofol with lidocaine, clove oil, and MS222) seemed to be equally valid regarding the avoidance of aversion and stress.

Implications for the next chapter

After this study, etomidate seemed to be more aversive than the other anaesthetics, thus we did not used it in further studies. Also, although clove oil had good results in this study, it was not tested further, as it needs to be solubilized in ethanol prior to immersion, having one more chemical to control, and it has a strong odour not being practical to use in the animal facility. For these reasons, we decided to study only the effects of MS222 and propofol/ lidocaine anaesthetics on memory (Chapter 4). These further studies will clarify if the lower aversive profile of MS222 and propofol/ lidocaine in comparison with etomidate is related to the amnesic properties of the anaesthetics.

Chapter 2 - Instruction manual for the assembly and use of the set up to perform the one trial inhibitory avoidance task for adult zebrafish

This chapter sections were formatted and ready to be submitted to the HardwareX journal.

Hardware in context

Consistent and simple behavioural paradigms are required to investigate learning and memory processes in zebrafish, an animal model increasingly used in neuroscience. Often, these learning paradigms were based on earlier research on the behavior of other species such as mice. Adult zebrafish have been shown to be able to learn various types of memory, including the association between a neutral or preferred stimulus (unconditioned) and an aversive stimulus (conditioned). The aversive stimulus can be an electric shock [134]) or confinement in a tight space [163], for example, and the unconditioned stimuli can be of various kinds, such as visual discrimination [164-166], spatial alternation [167], and spatial conditioning [134].

A simple learning procedure similar to those used in passive inhibitory avoidance or other quickly learned fear-conditioning protocols are required because the many memory tasks performed need lengthy training sessions that frequently span for multiple days and are based on shuttle box or active-avoidance protocols [134]. In this sense, it is quite hard to identify the different memory processes and the influence of other external variables. The one-trial inhibitory avoidance task solves this by being a rapid to learn test, where, with a mild-electric shock, zebrafish learn in only one conditioning session to avoid the conditioned context [134]. But for this, there is a need for a testing apparatus that may be expensive to acquire or adapt, and there is no detailed description in the literature about a plan to build and assemble it in-house. The availability of 3D printing and easiness to modify our testing hardware makes it easier to create and use these tests. To do so, we created our own zebrafish-focused apparatus, which keeps costs down and ensures reproducibility, while enabling the study of a variety of learning-based conditions.

Hardware description

Behavioural testing apparatus are commonly used in research, but they usually need to be purchase from a specialty company, where the products are commonly very expensive, or to be built in-house, which makes it more difficult for other laboratories to reproduced them. Our system is designed to be in between; a cheap alternative that can be built in-house in a reproducible way achieved by the detailed description published here in open access. There are 2 primary parts to this hardware: the shock device and the context tank.

Fish are evaluated in a context tank equally divided in different coloured walls to provide an environment with different contextual stimuli (black and white) to be distinguished. This can be directly compared to what can be purchased for about 1200 euros (e.g., Mazeenginners: https://conductscience.com/maze/portfolio/zebrafish-light-dark/). Unlike these tanks that can be purchased, whose walls can only be black and white, our tank's walls can be custom-coloured as each wall is an insert plate. Also, another commercially available apparatus

(https://zantiks.com/protocols/pavlovian-avoidance-learning) has already the shock unit in place, but the walls are always white, although different images/ colours/ textures can be projected in the tank floor. This company usually sells a package with several behavioural tests to be adapted, and not only one test, thus the price increases.
Our tank is far less expensive; assuming you have access to a 3D printer, the PLA will run you about €15. The water and the fish used were tested in a Tecniplast™ 1L tank inside of the plastic shell 3D printed in black and white. These tanks cost around 10 euros. The apparatus's dimensions can be altered to fit tanks of various sizes.

The variable bench power source unit (PSU), which accounts for most of the cost of the shock unit (85€), may be cheaper if choosing a version with smaller ranges (voltage and current). Additionally, the use of a fixed power source is an option since these plans also include a voltage divider component that can divide the voltage in drops of 1/12, offering a less expensive option at the expense of fine-tuning voltage.

This design's simplicity of use after assembly is almost identical to that of a store-bought version. The version presented here is a white and black box although it can be customized to use other colours to test other paradigms. This can be achieved by changing the colour of the walls thanks to being able to print your own walls and floor for the exterior plastic shell: This way, different aspects of aversion or preference of fish can be explored, which is often important in the context of neuroscience research. Summing, this design brings the following advantages:

- It is a modular hardware, allowing to change the colours of the walls and floor (contexts cues), enabling to study other paradigms of preference or other associative learning tests rather than only have the black/ white tank available.
- It is easy to assemble the plastic shell + shocking device, allowing a quick study of learning and memory performance.
- Considering the use of a variable power source unit, the easy manual selection of the voltage allows the adaptation of its value to the conductivity of the water used, or if you want to use another voltage to other aim. However, note that we have tested different properties of the electrical shock (Table 2.1) to be sure this is the minimal electric shock that the animal finds aversive; if it is aversive, the animal will learn to avoid it.
- The assembly of this apparatus has a low cost (~160€)

Voltage	Amperage	Number of shocks	Time under shock (ms)	Conditioned?	Number of animals
3	0.3	5	100	no	10
5	0.3	5	100	no	10
6	0.3	5	100	no	10
5	0.5	5	100	no	10
6	0.5	5	100	no	10
5	0.5	5	400	no	10
6	0.5	5	400	no	10
5	1	5	400	no	10
6	1	5	400	no	10
2.2	1	5	400	no	10
3	1	5	400	no	10
3	1	1	5000	no	10
3	1.5	1	5000	no	10
3	2	1	5000	yes	10

Table 2.1: Summary of all electrical parameters tested during our pilots until reaching conditioning conditions. Conditioning happens when the animal takes significantly longer to enter the side of the tank where it was shocked, 24 hours after the event.

Design files summary

Design file name	File type	Open source license	Location of the file
Structure of tank file	CAD	*CC-By Attribution 4.0 International*	OSF
Circuit file	Figure	*CC-By Attribution 4.0 International*	OSF and see annex 3
Code file	IDE file	*CC-By Attribution 4.0 International*	OSF and see annex 4
Assembly aid	Figure (zip)	*CC-By Attribution 4.0 International*	OSF and see annex 5
Circuit layout	Figure (PDF)	*CC-By Attribution 4.0 International*	OSF and see annex 6

Structure of tank file: CAD file with all the necessary components and dimensions to replicate our experimental tank.

Circuit file: image with all the necessary connections for our shock device component in an easy-to-read format.

Code file: code to be uploaded to the Arduino. Ready to use and customized. Commented for easiness of read.

Assembly aid: set of pictures of close ups of the experimental apparatus to help assembly if required.

Circuit layout: figure with the circuit layout presented in a technical manner.

Bill of materials summary

Designator	Component	Number	Cost per unit (€)	Total cost - (€)	Source of materials	Material type
Arduino	*Arduino*	1	24€	24€	https://tinyurl.com/y4nz86ta	Micro controller
Resistor	*Resistor 1kΩ*	14	0.1€	1.4€	https://tinyurl.com/5n7tfjwj	Other
Breadboard	Breadboard	2	0.99€	1.88€	https://tinyurl.com/489ejcxa	Other
Button	Button	1	0.36€	0.36€	https://tinyurl.com/h7ftvz2c	Other
Wire	Wire	1	3€	3€	https://tinyurl.com/yxm4n6em	Other
Relay	Relay	1	4€	4€	https://tinyurl.com/7s343swc	Other
LED	LED	2	0.1€	0.2€	https://tinyurl.com/bd5xyhj9	Other
Banana connector female	Banana connector female	2	2€	4€	https://tinyurl.com/af72ba4r	Other
Banana connector male	Banana connector male	2	4€	4€	https://tinyurl.com/2p8wvvw8	Other
Power source	Variable power source	1	84.9€	84.9€	https://tinyurl.com/ys94brwx	Other
Filament for 3D printer	PLA filament	1kg	20€	20€	https://tinyurl.com/55d7jtkn	plastic
Plastic Tank	Tecniplast 1L tank	1	10€	10€	Check with vendor	plastic

Build instructions

For the tank components:

- Start by opening the CAD files on a CAD software (e.g., Fusion 360) (File *Structure of tank file)*.
- If this is the design you want to print, just export each component separately to a .stl extension file. (divider, outer shell, inner shell, etc). If not, you can change the size of each component.
- Load each file on a 3D printing slicer software (e.g., Ultimaker Cura). Slice them choosing how fast you want the parts to be printed, how much plastic to use. We recommend a 10% filling with hexagon shaped structure. For faster printing, use triangle shaped inner structure. Then after slicing, you can send each file to the 3D printer for printing.
- Colour can be chosen from which filament is loaded on the 3D printer.
- Assemble each of the two outer shell parts by gluing them together (Figure 2.1) (e.g., using epoxy glue) and repeat the process for the inner shell parts. Do not glue the divider.
- Insert the male wall part into the corresponding female wall part and slide them together into the wall slot.
- Put the plastic tank inside the outer shell.
- Slide the female banana connectors into the inner shell holes and weld the wire to them or screw the wire tight depending on the type of connector you bought.
- Assemble electrodes from wire and loop them to connect to negative and positive wires of the PSU (black and red, respectively).
- The electrodes should be placed on the walls of the black compartment with oval loops being large enough to allow the fish to pass through it (2cm high at least) and width approximate to the size of the plastic tank.

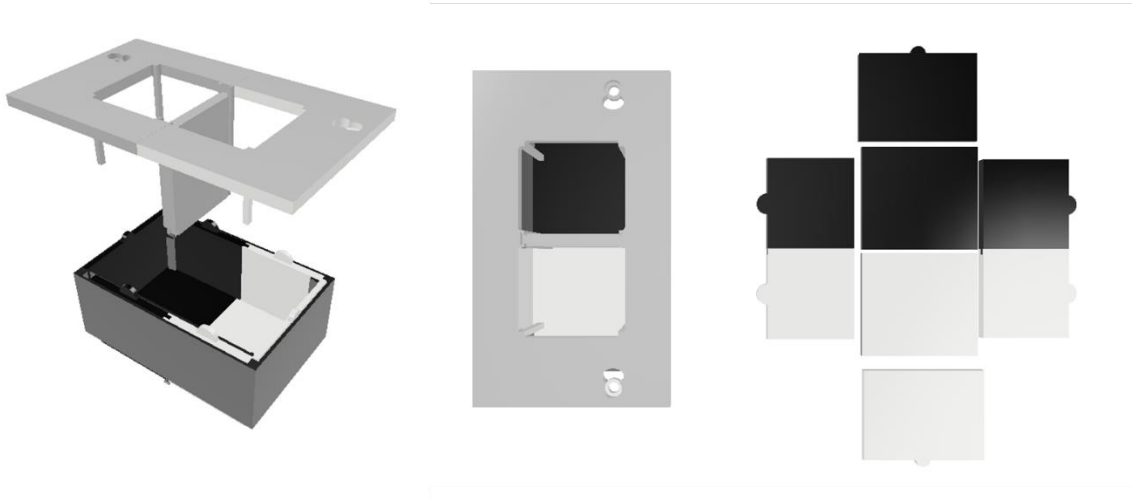

Figure 2.1: Schematic of the 3D printed apparatus in 3D rendering with an orthographic view (left), a top view (center) and a top view of the walls and floor separated from the apparatus (right). Holes and grooves on each side for electrode wiring and positioning and groove in the middle of the top part for the divider.

For the shock unit:

- Download Arduino IDE
- Upload Arduino code provided (see annex 3) into your Arduino, using a computer or a cell phone (ArduinoDroid or BLExAR/ Arduino pack for iOS, for example).
- Connect Arduino as shown in circuit layout (see annex 4).
- Assemble breadboard and components as shown in circuit layout file taking special attention to the led and button orientation (Figure 2.2)
- For variable PSU, connect positive wire to the 1/12 voltage drop and negative to ground.
- For fixed PSU, connect positive wire to the X/12 of voltage you want to use and then negative to ground. Due to the conductivity of the water, we recommend measuring the voltage in the water, in the black side, before behavioural testing with a multimeter or voltmeter.

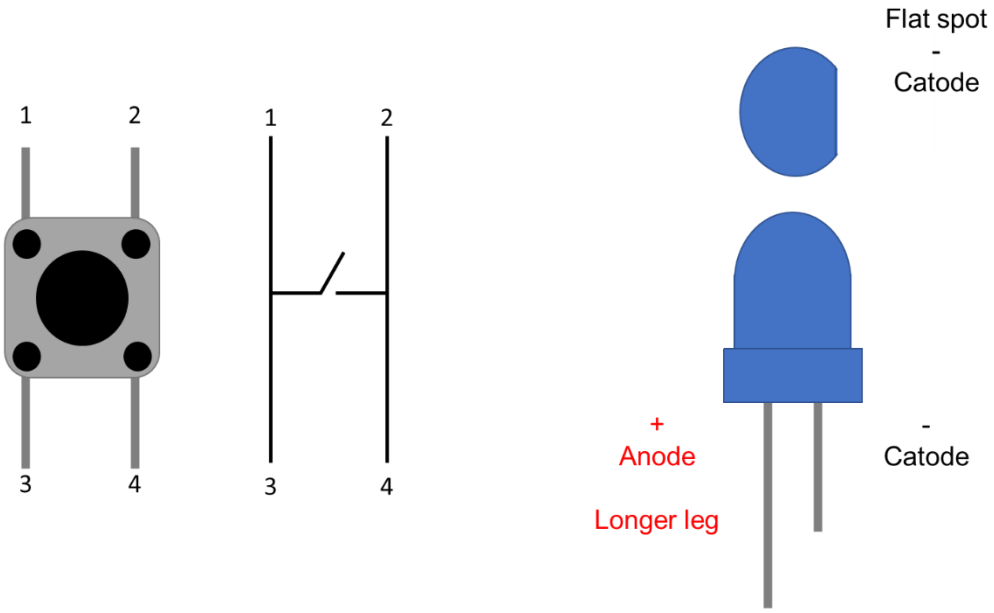

Figure 2.2: Button and Led schematics and further instructions. For the button, be wary that 1 and 3 are only connected to 2 and 4 once the button is pressed.

Operation instructions

- Connect both PSU and Arduino to a power socket.
- Connect the wires to the positive (red wire) and negative (black wire) plugs on the shocking device and the electrodes to the context tank (Figure 2.3).
- Submerge the electrodes in water by closing the shell.
- Check if red LED is on. If so, system is ready to use.
- Click the button to deliver shock. The second LED should turn on for the duration of shock, meaning, current is passing to deliver the shock.

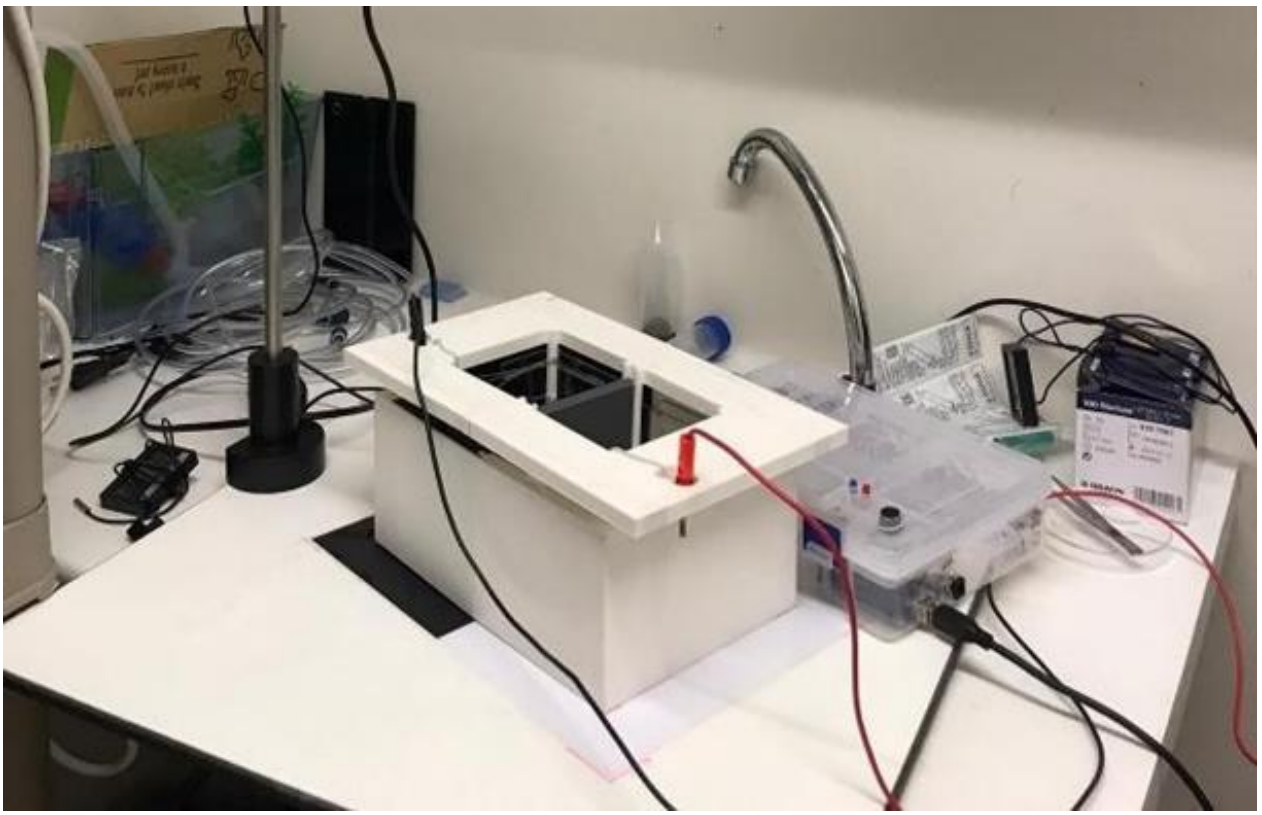

Figure 2.3: Assembled and connected apparatus

Validation and characterization

Neuroscientists frequently employ avoidance tasks to assess a variety of behaviours, including anxiety and learning [11, 22, 23]. These tasks evaluate the passive or active escape reaction, a general distress response that is important for self-preservation and is highly conserved [24]. Zebrafish from the strains used (AB and TU (described on the methods section on chapter 3)) initially show a preference for the black compartment in the present apparatus over the white compartment in pilots performed by our students. This preference may be explained by the possible aversion that white colour induces, as animals become more visible against that background, thus more exposed to threats. In this sense, animals are expected to quickly enter the black side. When this preferred side is conditioned with an actual aversive stimulus, such as electric shock, the animal is expected to avoid that compartment. Thus, 24h post-conditioning, the black side is perceived as aversive, but over time, the likelihood of another shock decreases, and anxiety levels influence the animal's decision-making to move there again [26]. The behavioural paradigm protocol is as shown in figure 4. Here, when we used an electric shock that induces aversion, the animal requires much more time to enter the black side, post-conditioning. This validates the set up and the shock as aversive, as the animal showed to remember the shock by avoiding the previously preferred side.

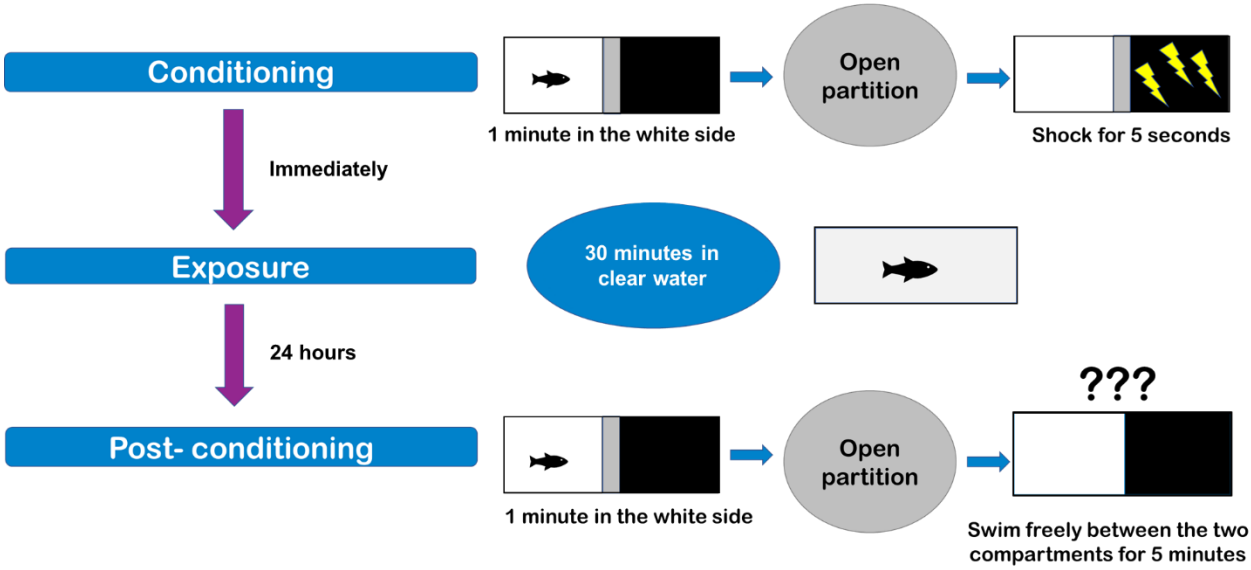

Figure 2.4: One-trial Inhibitory Avoidance Task behaviour scheme. Animals from two strains were subjected to the same procedure. After the shock in the black side - conditioning, they were individually placed in a tank with clean water for 30min. 24h later, the test is repeated without the shock. If they remember, the latency to enter in the black side would be higher after the conditioning.

Table 2.1 shows the several electrical parameter combinations that were tested, but only 3V with a current of 2A for 5 seconds produced consistent conditioning (figure 2.5).

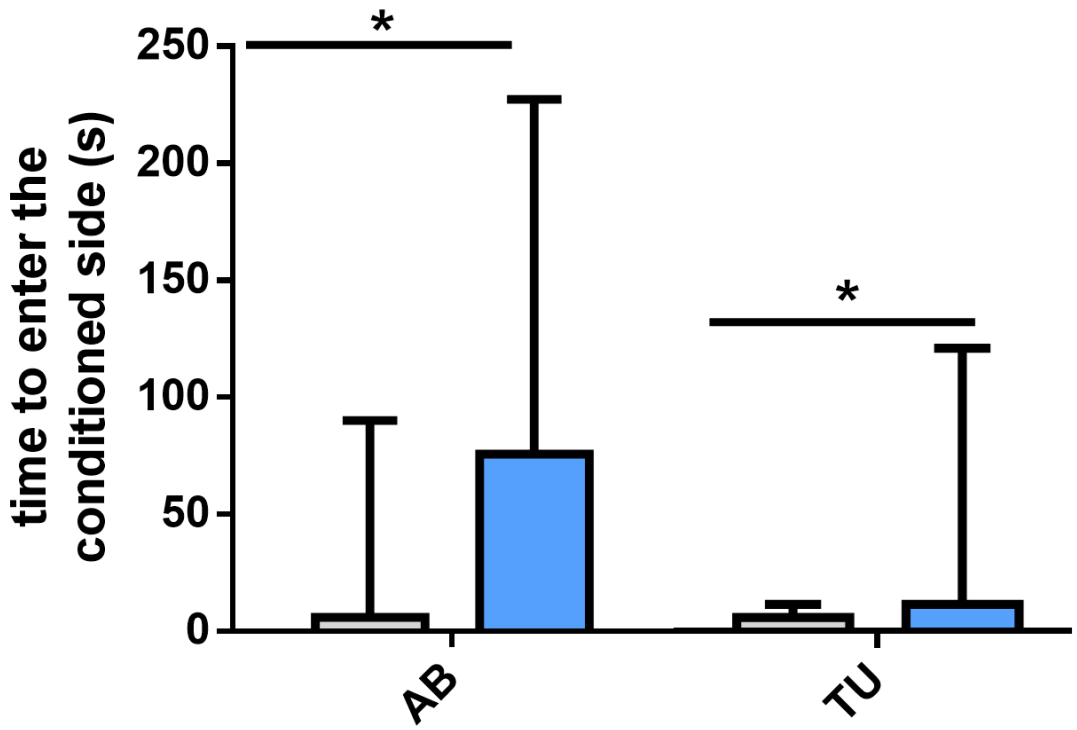

Figure 2.5: Latency to enter in the conditioned side in the conditioning phase and the post-conditioning phase for each strain. Control group: animals subjected to clean water (n= 20 for AB and 13 for TU). AB: animals from the WtAB strain, TU: animals from the WtTU strain. Data presented as median + IQR. * represents differences between phases within each strain (p≤0.039) using Wilcoxon signed-rank test.

Furthermore, the animal's ability to remember past unpleasant experiences is crucial for performing the task and avoiding future electric shocks, underscoring the importance of learning and memory processes. So, this apparatus has the potential to be used to study the effects of diseases, drugs or other environmental variables on memory and learning capabilities.

Capabilities of the hardware

- To assess aversion of different factors and different timings.
- This hardware and protocol allow to successfully test conditioned learning of the most used strains in laboratory zebrafish.
- To test preference for other colours or patterns.
- To test how drugs can modify the experience of the animal inside the apparatus, especially related to memory process and anxiety.

Limitations of the hardware:

- Other conductivities were not studied so might perform differently if conductivity is not 900 µS, and voltage must be adapted.

- Response might be age dependent due to the way current travels through different body composition (e.g., younger animals being leaner and older animals being fatter).

Implications for the next chapter

From this study, we designed a working apparatus in which the shock was validated, i. e., it produced enough aversion to revert the preference for black after being conditioned. This makes this apparatus useful to study learning/ memory in one conditioning trial. Following, we needed to validate if these results really mean that animals learned the shock by using a positive control in the study of the next chapter.

Chapter 3 - Validation of open source set up for one trial inhibitory avoidance task to test memory performance

This chapter includes material published as a preprint in bioRxiv:

Jorge M. Ferreira, Joana Silva, Sofia Barros, Ines Caetano, Pedro Fernandes, Anna Olsson, and Ana M. Valentim “Validation of the in-house designed one-trial inhibitory avoidance test for two strains of adult zebrafish” bioRxiv (2023)

Introduction

Creating, refining, and replicating protocols are key components of science. With the increase of zebrafish (*Danio rerio*) use as an animal model, accounting for 5% of all the animals used in the EU [41], there is an additional need for new and validated protocols that can be replicated. For a method to be replicable, it is crucial that it is properly reported in the literature, with all the details described. Failing to do so leads to the reproducibility crisis [168] and the waste of animals, which is an issue for the scientific results and for the 3Rs [42].

Often behavioural protocols tested in rodents are adapted to zebrafish, as they have become a relevant model in different areas of research. It is rather important to correctly adapt these tests from rodents to zebrafish as they are different species with totally different needs. For instance, when adapting the white/black box protocol from rodents to zebrafish, there will be notable differences in the outcomes, especially because zebrafish are diurnal fish while rodents are nocturnal mammals. The white/black box is a set-up often used in the memory task passive avoidance [169]. In this test, also known as inhibitory avoidance test [170], the animal learns to avoid an unpleasant stimulus by limiting its locomotion and exploration. For zebrafish, this test was adapted [134] using a black and white tank where the animal will receive a mild shock in the black side of the box (their preferred side [171]) which they should learn to later avoid. To successfully replicate this task, a complete report of the shock profile is needed. The problem is that, although several articles report the voltage used and the duration of the shock, no report on the intensity of the shock (current in Amperes) was present. This is problematic as a shock relies on four major components: intensity, current, duration and type of current [172]. If information about one of these components is missing, it is impossible to assess how strong the shock was and impossible to replicate the method. Also, there is great variations for the components that are reported in the literature, with voltages ranging from 3 and 12 volts, duration of the shock from 1ms to 5000ms and currents being AC or DC [134, 173-178], thus standardization is required. Another issue for implementing this task is the lack of a commercial version of the apparatus; and the ones that could be adapted were very expensive. Therefore, our objective was to create and validate an affordable open source custom-made apparatus and protocol that allowed testing the memory of the two most used strains of zebrafish (AB and TU [179]). Indeed, different strains have been describe to exhibit different behaviours [178, 179].

The validation of the test was pharmacological, using a known amnesic as a positive control, the NMDA receptor antagonist MK-801. As an amnesic drug [180, 181], animals treated

with this substance are expected to show memory impairment, indicating that the test measures what it is supposed to evaluate, memory.

Material and methods

Ethics statement

All procedures were carried out under personal and project licenses approved by the National Competent Authority for animal research (Direção-Geral de Alimentação e Veterinária, Lisbon, Portugal) (approval number: 014703), and by the Animal Welfare and Ethics Review Body of the Institute for Research and Innovation in Health, according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and its transposition to the Portuguese law, 'Decreto Lei' 113/2013.

Animals and Housing

Sample size calculation was performed in G*Power 3.1 (University of Düsseldorf, Germany), assuming type II error probability of α=0.05, a power of 0.85, and an effect size of 0.730, based on previous data (pilot study). Adult (7-16months old) mixed-sex zebrafish (N=72; 40 for AB + 32 for TU) bred in the Animal Facility of i3S were used. They were kept in groups in 3.5 L tanks (maximum of 8 fish per liter), in a 14h:10h light:dark cycle, in a recirculating water system connected to a central unit of water purification and controlled conditions (27 ± 0.5 °C, pH of 7 + 0.5, conductivity of 800-900 μS). Adult fish were fed three times a day with a commercial diet (Zebrafeed 400-600 μm, Sparos, Olhão, Portugal). The same conditions of water were kept during the behavioural test.

Treatments

Zebrafish were randomly distributed to the control and amnesic treatment (positive control) group in each strain tested. Control group (N=37, 20 AB, 17 TU) was exposed to clean water, while amnesic treatment animals (N=35, 20 AB, 15 TU) were exposed to 20μM of MK-801 (MK-801 hydrogen maleate, M107, Sigma–Aldrich, USA) for 30 minutes.

Behavioural test - One trial inhibitory avoidance task

For this test, a modified passive avoidance task, based on the approach described by Blank et al. [134] to study memory and learning deficits was used. Our test is carried out in an apparatus that consists of a transparent tank surrounded by a 3D printed shell that is half white and half black with a moving barrier in the middle that can be raised or lowered manually allowing animals to visit both sides of the tank (Figure 3.1); this was connected to

a shock device (Figure 3.2) that modulates the shock from a power source (building instructions in Chapter 2). This paradigm consists on pairing an aversive experience with a previously neutral/preferred place [182]. The degree of avoidance is directly related to the latency of entering the previously preferred side and the time spent on each side after conditioning [7].

Set-up

For the experiment, a custom-built 3D printed apparatus and custom-made hardware and software were made for the shock unit (Figure 3.1 and 3.2 and Chapter 2). All test sessions were filmed from above with a camera (25 fps, Canon Legria HF R606, Ōta, Japan) that was fixed to a metal support at 45 cm of the apparatus, making sure no shadows were covering it.

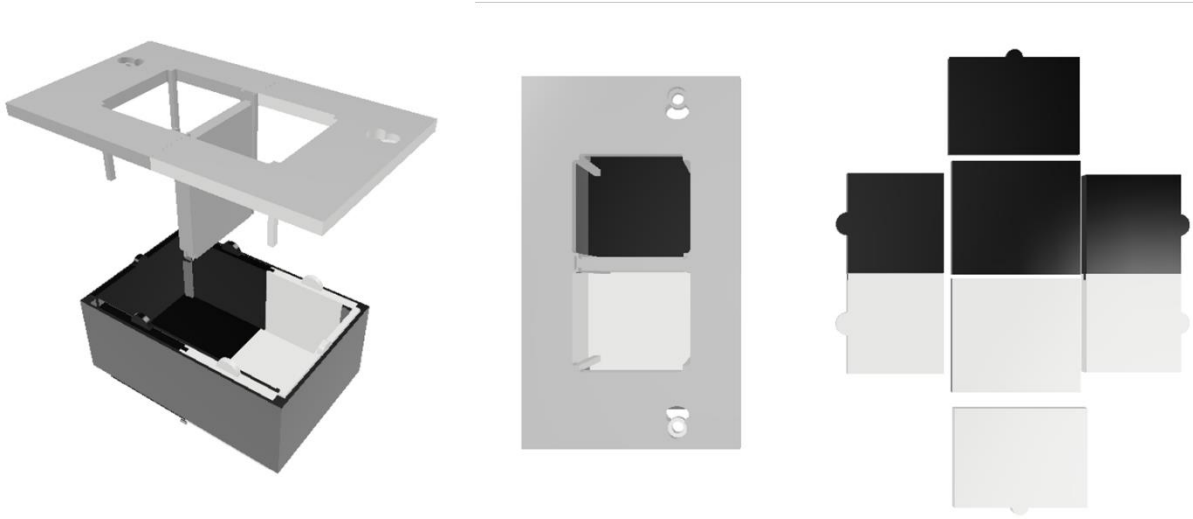

Figure 3.1: Schematic of the 3D printed apparatus in 3D rendering with an orthographic view (left), a top view (center) and a top view of the walls and floor separated from the apparatus (right). Holes and grooves on each side is for electrode wiring and positioning, and the groove in the middle of the top part is for the divider.

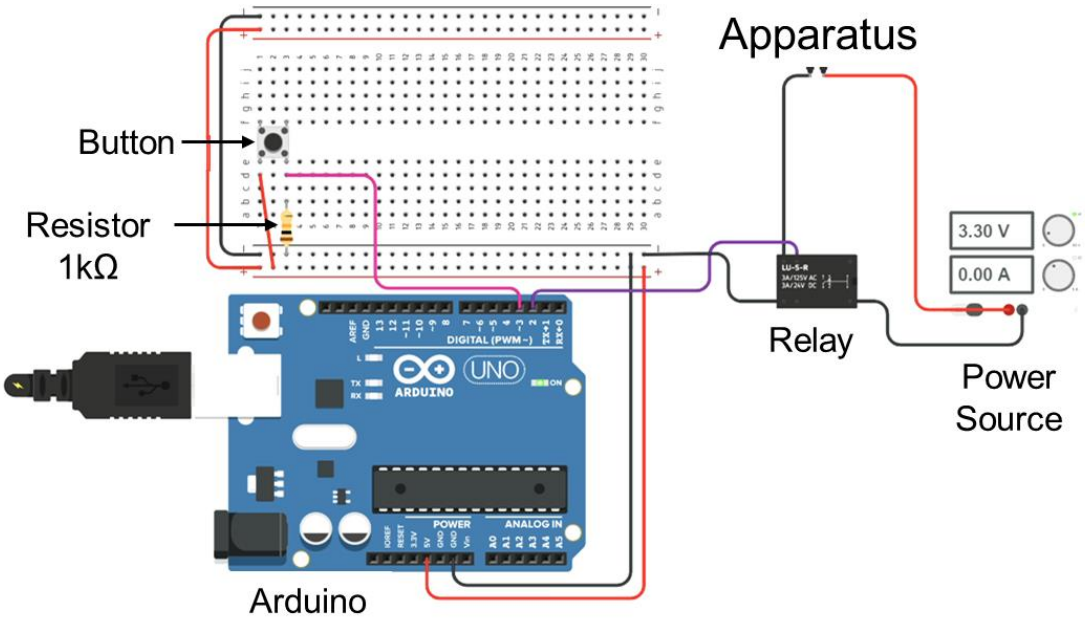

Figure 3.2: Schematic of the circuit layout of the shock unit. When the button is pressed, the shock is delivered by closing the electrical circuit.

Apparatus

This equipment consisted of an apparatus (Figure 3.1) with two equally sized black and white compartments. The apparatus has three parts: a Tecniplast ^{TM} plastic 1L breeding tank (16.5cm x 9.5cm), a shell that provides the desired colours to the apparatus (outer layer), and a shell that holds a divider and provides support for the electrodes (inner layer). The division of the compartments is done using a removable grey barrier with a thickness of 1 cm. The tank was filled with 300 ml of the recirculating water system (at 900 µS) where the zebrafish were housed, providing a 3 cm water column.

Shock unit

This component consists of a direct current (DC) power supply (UNI-T, UTP1305, Uni-Trend Technology, Dongguan City, Guangdong), connected to a relay controlled by an Arduino UNO (Arduino, Ivrea, Italy); see annex 6 for circuit layout and chapter 2 for instructions. We choose to use a DC constant current, instead of an alternating current because it is the most commonly used for electronic circuits while being the one allowing more control.

One-trial Inhibitory Avoidance test

The schematic representation of this behavioural protocol is showed in Figure 3.

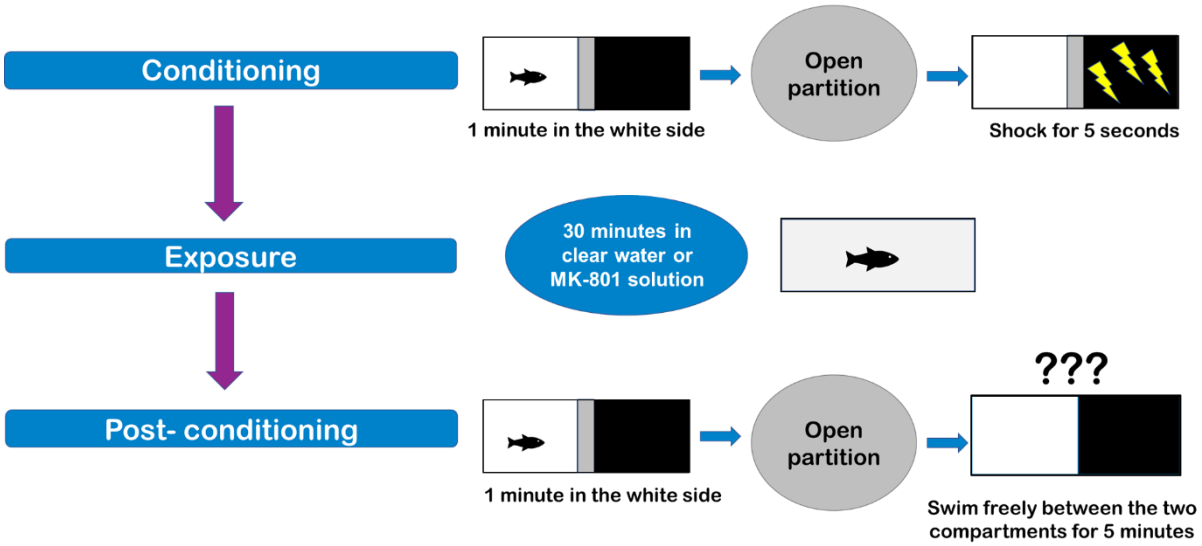

Figure 3.3: One-trial Inhibitory Avoidance Task scheme. Control and MK-801 groups were subjected to the same procedure, except they were individually placed in a tank with clear water or 20µM of MK-801 solution, respectively, for 30min.

Conditioning session

Individual fish were carefully placed on the white side where they remained for 1 min before the barrier was lifted. This was done to increase the ability of the animals to associate behaviour-consequence during memory acquisition, in which temporal relations play an important role [183]. Thereafter the barrier was lifted 1 cm from the bottom of the tank, and immediately closed as soon as the animal crossed to the black side where an electric shock (3.3 ± 0.3V and 2A) was administered for 5 seconds after closing the barrier. Immediately after, the individual was then removed from the apparatus and placed in an amnesic bath of 20 µM of MK-801 (MK-801 group) or in clean water (control group) for 30 min. The animals were then allowed to recover individually in clean water until the next day.

Post-conditioning session

Twenty-four hours after the conditioning session, animals went back to the apparatus, and they were again retained for 1 min in the white zone. Subsequently, the barrier is lifted, and the animals were then able to swim for 5 minutes in the entire apparatus. Latency to enter the black side, latency to reenter the white side, time spent on each side and frequency of crossings were measured.

The entrance in one compartment was recorded when all the body entered in the new compartment and stayed there for more than 5 frames.

Different electric shock characteristics (voltage and amperage) were preliminary tested until reaching the values presented here (Chapter 2). First, low current and intensity were tested, and these were increased until the animal found the shock aversive, by avoiding it.

Video Analysis

All videos were analysed by Ethowatcher, a free event-coding software developed by researchers from the Federal University of Santa Catarina, Brazil [147]. The analysis was carried out by an experienced researcher blind to the treatment, and two other blinded researchers confirmed the results by redoing the analysis.

Data Analysis

Data was analysed using IBM SPSS™ 26 for Windows (SPSS Inc., Chicago, IL, USA) after exporting it to the Microsoft Excel™ 2010 (Microsoft Corporation, Redmond, WA, USA). First, data were analysed to check their normality (Shapiro-Wilk test) and homoscedasticity, that is, homogeneity of variances between groups using the Levene test. If these assumptions were fulfilled, a parametric test was used; otherwise, the analysis was performed with a non-parametric test. For the parametric tests, a paired Student's t-test (which compares means of continuous dependent variables between two related groups) was used for comparisons within groups, while the Wilcoxon signed-rank test is the correspondent nonparametric test. These tests were used to compare the differences within each group on the latency to enter the black side before and after the electric shock, and on the time spent between both sides of the apparatus during post-conditioning.

For comparisons between groups, an independent Student's t-test was used for parametric data, and a Mann-Whitney U test was used for nonparametric data. These tests compared the differences between groups regarding the latency to enter the black side in the conditioning and post-conditioning session, the time spent on each side of the apparatus, and the frequency of visits to the black side during post-conditioning. All hypotheses were two-tailed tested and statistical significance was set at $p \leq 0.05$.

Results

Animals

Two animals from the AB strain of the MK-801 group had to be eliminated from the analyses because there was a failure to close the barrier when individuals were introduced to the white side of the apparatus in the conditioning phase.

Treatments modulate the response of zebrafish after exposure

Latency

First, in the conditioning phase, we checked if the animals behaved similarly before the treatment was applied; indeed, the latency to enter the black side were similar between control and MK-801 group, and there were also no strain differences.

An animal that has learned and remembers the location of the aversive electric shock will present a higher latency to enter the black compartment after conditioning. Both control animals of the AB and TU strain took longer to enter the black side in the postconditioning compared with the conditioning phase. ($Z=-2.315$, $p=0.021$ and $Z=2.062$, $p=0.039$ for the AB and the TU strain, respectively). Both strains treated with the MK-801 did not show differences in this variable (Figure 3.4).

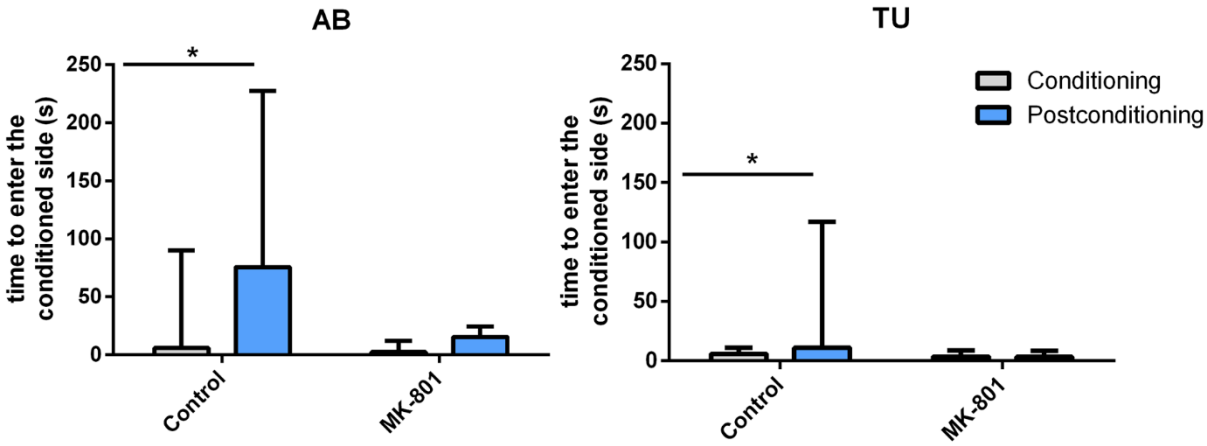

Figure 3.4: Latency to enter in the conditioned side (black side) in the conditioning phase and in the post-conditioning phase for each treatment group and each strain. Control group: animals subjected to clean water (n= 20 for AB and 13 for TU); MK-801 group: animals subjected to 20 µM of MK-801 for 30 minutes (n= 20 for AB and 15 for TU). AB: animals from the WtAB strain, TU: animals from the WtTU strain. Data presented as median + IQR. * represents differences between phases within each group (p≤0.039) using Wilcoxon signed-rank test.

Time spent in each side

Regarding the time spent in each side of the apparatus during the post-conditioning phase, the animals from the MK-801 group from both strains spent more time in the black side (AB: Z=2.678, p=0.007 and TU: Z=2.897, p=0.004). In the control group, animals from the TU strain control group spent more time in the black side (Z=2.271, p=0.023), whereas AB strain control showed no side preference (Figure 3.5).

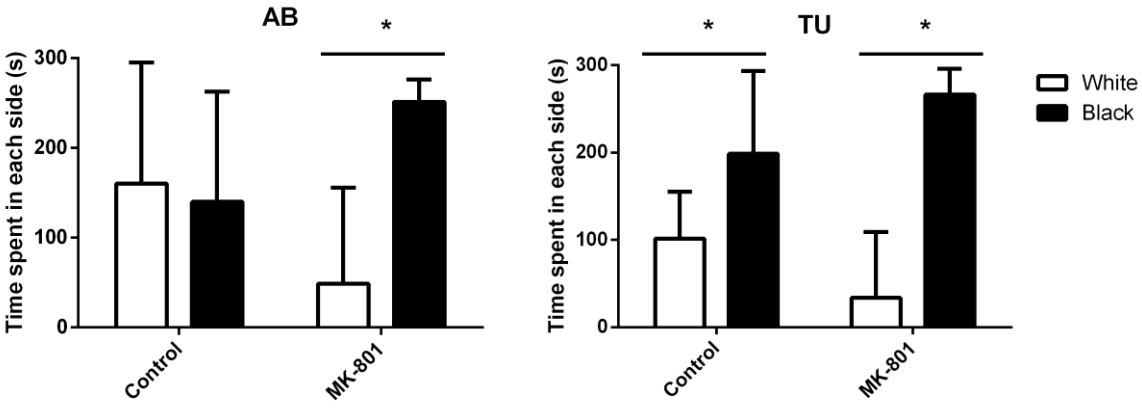

Figure 3.5: Time spent in each side of the apparatus in the post-conditioning phase for each treatment group and each strain. Control group: animals subjected to clean water (n= 20 for AB and 13 for TU); MK-801 group: animals subjected to 20 µM of MK-801 for 30 minutes (n= 20 for AB and 15 for TU). AB: animals from the WtAB strain, TU: animals from the WtTU strain. Data presented as median + IQR. * represents differences between sides of the apparatus within each group for each strains ($p \leq 0.023$) using Wilcoxon signed-rank test.

Frequency to visit each side

The frequency to visit the black side after the conditioning was reduced in the control group compared with the MK-801 group in the AB strain ($U= 261.0$, $p=0.017$); however, this difference was not detected in animals from the TU strain (Figure 3.6).

Although some results were different for the AB and TU strain, there were no differences detected between strains related to control and MK-801 group in the post-conditioning phase.

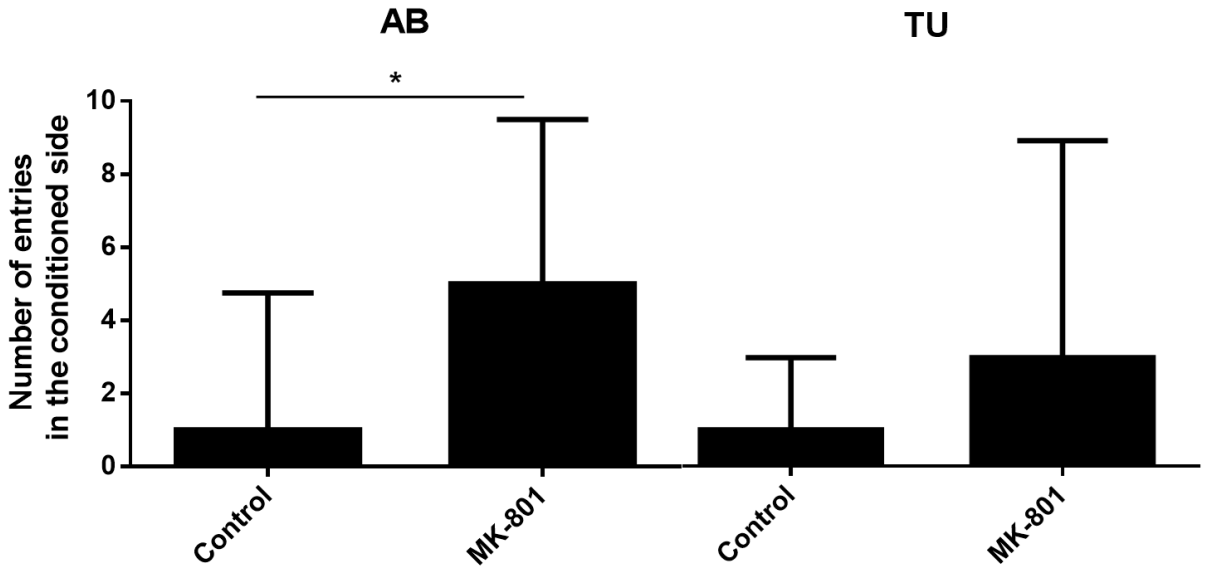

Figure 3.6: Number of entries in the conditioned side (black side) of the apparatus in the post-conditioning phase for each treatment group and each strain. Control group: animals subjected to clean water (n= 20 for AB and 13 for TU) ; MK-801 group: animals subjected to 20 µM of MK-801 for 30 minutes (n= 20 for AB and 15 for TU) . AB: animals from the WtAB strain, TU: animals from the WtTU strain. Data presented as median + IQR. * represents differences between groups in the AB strain ($p<0.001$) using Mann-Whitney test.

Discussion

When considering a new behavioural test or apparatus to use, replicability and reproducibility are of utmost importance as they are key points for our data to be compared with the ones from literature. In addition, accessibility and cost are two components that allow others to reproduce and replicate our data. This is important as then we can further create more accurate experiments and improve the standardization of the methodologies. Unfortunately, there is a methodology report crisis [168] mainly because the methods are not fully described, which leads to difficulties in replicating experiments. The test implemented in this work is an example of that, as the type of current and amperage was often not reported. It is essential to know the amperage to use, as the value determine the intensity of the shock. Additionally, there was also no fully available commercial version. Thus, we needed to build and test/ validate our own system. To validate this test, the electrical shock had to create sufficient aversion for animals to avoid the conditioned side after just one trial, as in Blank's work [134]. Additionally, the introduction of a drug known to interfere with memory would have to translate into data showing that treated individuals do not remember the shock, i. e., they should have the same latency to enter to the conditioned side before and after being subjected to the shock (conditioning). With both results being positive, we can say with confidence that our apparatus works.

In this study, using an apparatus fully assembled by us with refined parameters to the electrical shock, animals from both strains behaved similarly to the ones used by Blank and colleagues [134]; the control animals took longer to enter the black side after conditioning. Our MK-801 animals took the same time to go to the black side before and after the shock, meaning that the animals did not remember the shock, therefore not displaying learned aversion to the black side. These results already show two important premises: the shock had induced enough aversion in the control animals of both strains, and the NMDA antagonist disrupted the memory of the electrical shock in both strains.

Different zebrafish strains may have different behavioural profiles, which may result on different results of the test we want to validate [184]. However, we found no differences between the TU and AB strains tested in each measured variable. There were also no differences in preference for the black side when comparing both strains. We tested their preference in pilots performed before this study and a consistent preference for the black side was in place. Nevertheless, the behavioural profile of each strain was different with respect to the time spent in each side of the tank and the frequency of entries in the black side after being conditioned. During post-conditioning, the AB control did not reveal a preference for any side of the tank, while AB MK-801 group spent more time in the black side. These results are in line with the ones from the latency to enter the black side, where

control animals showed avoidance for the black side, but not the MK-801 animals (impaired memory). On the contrary, the TU control animals spent more time in the previously preferred place, the black side. It has been described that TU strain recovered faster its normal activity in the novel tank test, indicating less anxiety-like behaviour in space occupation than the AB strain [179], which could explain the results obtained. Thus, the TU control may have low levels of anxiety compared with AB control, allowing them to recover from the previously unpleasant experience and spending more time in the black side (the preferred side) where they no longer receive an electric shock. Similarly, AB control entered less in the black side than the AB MK-801 group, while the MK-801 and control group from the TU presented equal number of entries in this side. These results did not indicate that the TU strain did not learn, as they remembered the shock, showing longer time to enter the black side after conditioning, as referred. Also, the amnesic group MK-801 was only different from the control group in both strains in the latency to enter the black side. Thus, the latency to enter the black side before and after conditioning appears to be the most important variable to evaluate memory in this task, while the others may be influenced by anxiety.

Except for the frequency to enter the black side, both strains had the same response to the amnesiac drug, showing that the NMDA receptors were similarly affected by MK-801, and both strains forgot the aversive experience.

Avoidance tasks are very important in neuroscience as they have been used for a variety of assessments, from learning to anxiety [164, 167, 175]. This is based on the assessment of a nonspecific distress response, the escape response, which is a key behaviour for the survival of species, and thus a well conserved trait [185]. In this test apparatus, initially, the zebrafish have the tendency to avoid the white side, preferring to go to the black compartment, while the white colour may expose the animals to potential threats; different anxiety levels may modulate this behaviour. Later (post-conditioning) the animal must passively avoid a real aversive stimulus, not going to the previously preferred place, where fear may play its role. Thus, here, fear is potentially triggered but fades during the post-conditioning phase and the animal enters the black side. In this sense, anxiety may also be involved in the animals' decision [186].

Linked with this, the animal needs to remember previous unpleasant experiences to perform the task and avoid another potential electric shock; for that, learning and memory processes are key. Since memory is mediated by synaptic plasticity [187, 188], we can look at neurotransmitter receptors and modulate them with amnesic drugs such as the *N*-methyl-D-aspartate (NMDA) receptor, which has been reported to be involved in fear-avoidance learning [186]. Previous studies in zebrafish and other species [134, 189-191] have shown

that MK-801 is effective in preventing memory formation when given after training in similar inhibitory avoidance tasks [134, 191].

Fortunately, developments in 3D printing technology made the building of this type of test very affordable and the tools are easy to access when paired with the availability of open-source hardware microcontrollers. This inexpensive easy to assemble apparatus can be used to perform a reproducible task for memory and learning assessment in adult zebrafish from both AB and TU strains, as we provide here and in (Chapter 2) all the details to build the test, and a pharmacological validation.

Implications for the next chapter

This study validated that memory could be studied using the described paradigm of inhibitory avoidance developed and tested. Also, we showed that it is possible to assess memory/learning impairments from drug exposure using this behavioural test; in this sense, it is ideal to test anaesthetic effects.

Chapter 4 - Do MS222 and Propofol/ Lidocaine produce an effect on the learning performance and brain proteome of adult zebrafish?

Introduction

Although anaesthesia is extensively used, its mechanisms of action still need to be fully understood. The potential side effects are vastly unknown as a wide range of drugs is used to produce unconsciousness [192]. One of the most described effects associated with surgery and anaesthesia is the postoperative cognitive dysfunction (POCD), which has been described in humans and other mammals [4, 22, 23, 32]. Anaesthetics such as propofol interfere with multiple targets, but it is better known to decrease neuronal activity by enhancing the activity of the GABA-A receptor, leading to increased inhibition and decreased communication between neurons [193, 194]. In contrast, the local anaesthetic lidocaine has been described to induce retrograde amnesia [195] by blocking sodium channels in neurons, disrupting memory consolidation and recall.

Studies have shown that exposure to anaesthesia alters the levels of specific brain proteins and neurotransmitters and increases several inflammatory markers in the brain [34]. However, it is unknown whether cognitive decline presented after surgery is directly related to anaesthesia interfering with the memory processes or can also be attributed to neuroinflammation [196].

The prevalence of POCD is not well established in rodents, but studies have reported alterations in behaviour and cognition after individuals were anaesthetised [4, 5]**.** As for zebrafish, little is known, and there is a need to understand this phenomenon better because, in addition to the clinical implications, side effects of anaesthesia can also interfere with the research outcomes.

This study aims to determine whether anaesthesia influences the behaviour and synaptic protein expression of zebrafish after a learning event followed by an anaesthetic episode. This can allow us to isolate the memory component from an inflammatory event, giving us more information about the theory that the inflammation associated with surgery is the triggering factor and not the synaptic proteins that are affected. For that, our target anaesthetics is the most used anaesthetic (MS222) in zebrafish and the combination of propofol and lidocaine (both anaesthetics used in clinical settings).

As the proteome is quite conserved across species, the expected results may provide useful information regarding neurochemical and behavioural processes, having an impact in the clinical field, but also in research by providing vital information to minimise anaesthesia side effects both in humans and animals.

Material and methods

Ethics statement

All procedures were carried out with personal and project licenses approved by the National Competent Authority for animal research (Direção-Geral de Alimentação e Veterinária, Lisbon, Portugal) (approval number: 014703) and by the Animal Welfare and Ethics Review Body of the Institute for Research and Innovation in Health. Therefore, all experimental procedures were carried out in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and its transposition to the Portuguese law, 'Decreto Lei' 113/2013.

Animals and Housing

Sample size calculation was performed in G*Power 3.1 (University of Düsseldorf, Germany), assuming type II error probability of α=0.05, a power of 0.85, and an effect size of 0.730, based on previous data (from chapter 2). Adult (6-8 months old) mixed-sex AB zebrafish (N=89) bred in the Animal Facility of i3S were used. They were kept in groups in 3.5 L tanks (maximum of 8 fish per litre), in a 14h:10h light:dark cycle, in a recirculating water system connected to a central unit of water purification and controlled conditions (27 ± 0.5 °C, pH of 7 ± 0.5, conductivity of 800-900 μS). The fish were fed three times a day a commercial diet (Zebrafeed 400-600 μm, Sparos, Olhão, Portugal) for adults.

Treatments

Zebrafish were randomly distributed to the control and anaesthesia groups to be tested in experiment 1; Control group (n=20) was exposed to clean water for 10 min, MS222 group (n=20) was exposed to150 mg/L of MS222 buffered to pH 7 for 10 min (MS222, Sigma-Aldrich, Burlington, MA, USA); P/L group (n=20) was exposed to 5 mg/L of propofol and 150 mg/L of lidocaine for 10 min (propofol, Lipuro 2%, B. Braun Melsungen AG, Germany; and lidocaine, 2%, Braun, Queluz de Baixo, Barcarena, Portugal). Additionally, a naïve group was included for the proteomics analysis (n=5) with animals picked from their home tanks. For experiment 2; control group with revisit (n=6) where animals are subjected to a post-conditioning phase, control group without revisiting (n=6) where animals are not subjected to a post-conditioning phase, MS222 group (n=6) where animals are not subjected to a post-conditioning phase, and propofol/lidocaine group (n=6) where animals are not subjected to a post-conditioning phase.

One-trial inhibitory avoidance task

For this test, a modified passive avoidance task, based on the approach described by Blank et al. [134] to study memory and learning deficits was used and previously validated by us in (Chapter 2 and 3). Our test is carried out in an apparatus that consists of a transparent tank (16.5 cm X 9.5 cm, 300 mL of system water) surrounded by a 3D printed shell that is half white and half black with a moving barrier in the middle that can be raised 1 cm or lowered, allowing animals to visit both sides of the tank (Figure 3.1). This paradigm consists on pairing an aversive experience with a previously neutral/preferred place [182]. The degree of avoidance is directly related to the latency of entering on the previously preferred side (black side) and the time spent on each side [7].

In this study, after one min in the white side, the barrier was lift and the animal entered the black side where the aversive stimulus electric shock (3V ± 0.3V DC; 2A) was applied for 5 s conditioning phase. Then, the treatment described above was applied randomly distributing the animals per groups: control, MS222 or P/L. Afterwards, the animal r
in clean water until the next day. After 24h, the zebrafish was placed again in the w
of the apparatus, and the protocol was repeated without the shock and the animal was allowed to explore the whole apparatus for five min – post-conditioning phase (Figure 4.1). All videos were recorded at 25 fps and analysed by Ethowatcher (free, developed by researchers from the Federal University of Santa Catarina, Brazil) [147], where we recorded the latency to enter in the black side in both phases and the time spent in both sides in the post-conditioning phase. The animal was considered to enter in one of the sides when no part is visible on the white side for more than 5 frames. The researcher analysing the videos was blinded to the treatments.

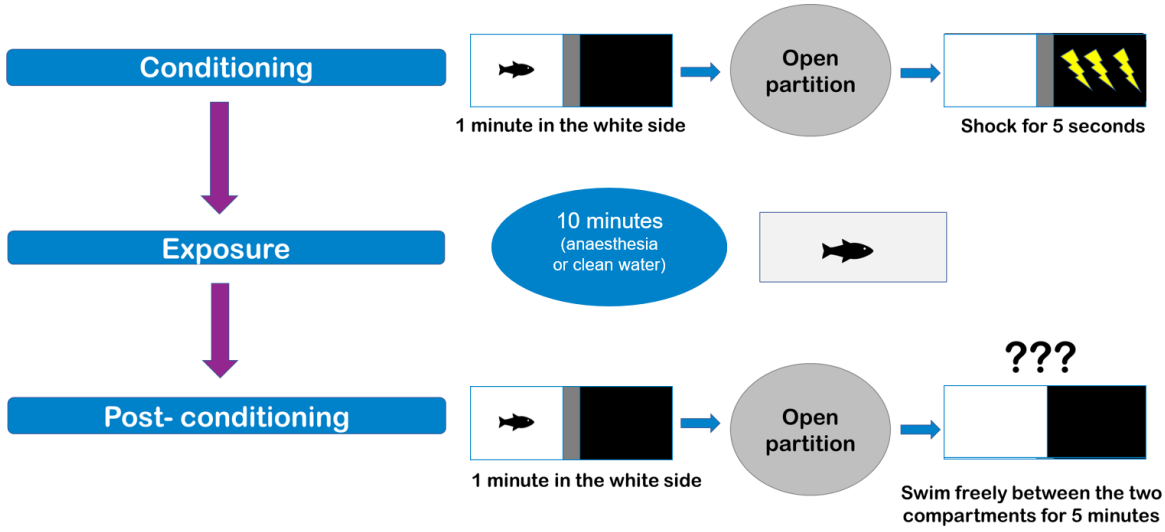

Figure 4.1: One-trial Inhibitory Avoidance Task scheme. In experiment 1 Control and anaesthetic groups were subjected to the same procedure, except they were individually placed in a tank with clear water or 150mg/L of MS222 or 5 mg/L of Propofol and 150 mg/L of Lidocaine combined, respectively, for 10min. For experiment two, the same was repeated but for the group CNS, and the anaesthetic groups, the post-conditioning phase was not performed.

Brain Processing

After euthanising the animals in a 2-4ºC water bath (rapid cooling), brains where dissected, flash frozen in liquid nitrogen and the whole brain was homogenised mechanically using an electrical mixing homogeniser and the synaptosome fraction was retrieve using Syn-PER according to the protocol provided by the manufacturer (Syn-PER, ThermoFisher, Catalog # 87793). Synaptosome fractions were store at -80ºC until being processed (Annex 7 shows data showing that extraction protocol was successful).

Quantitative proteomic analysis of synaptosomes

For S-Trap digestion, samples were alkylated with 10mM iodoacetamide in the dark for 30 minutes at 37°C. Triethyl ammonium bicarbonate was added to a final concentration of 50 mM, and protein was trapped and washed on S-trap micro spin columns (ProtiFi, LLC) according to the manufacturer's instructions. Protein was digested using 2.5 mg trypsin sequence grade (Pierce) at 47°C for 1 hour and 37°C for 1 hour.

For mass spectrometry (MS), eluted peptides were dried in a vacuum concentrator and samples were re-suspended in 40 µl of 0.5% formic acid, and 18 µl was analysed by nanoflow liquid chromatography–mass spectrometry using an Orbitrap Elite (Thermo Fisher) hybrid mass spectrometer equipped with a nanospray source, coupled to an Ultimate RSLCnano LC System (Dionex) at the biOMICS facility at the University of Sheffield. The system was controlled by Xcalibur 3.0.63 (Thermo Fisher) and DCMSLink (Dionex). Peptides were desalted on-line using an Acclaim PepMap 100 C18 nano/capillary BioLC, 100A nanoViper 20 mm x 75 µm I.D. particle size 3 µm (Fisher Scientific) at a flow rate of 5 µl/min and then separated using a 125-min gradient from 5 to 35% buffer B (0.5% formic acid in 80% acetonitrile) on an EASY-Spray column (50 cm × 50 µm ID, PepMap C18, 2 µm particles, 100 Å pore size (Fisher Scientific)) at a flow rate of 0.25 µl/min. The Orbitrap Elite was operated with a cycle of one MS (in the Orbitrap) acquired at a resolution of 60,000 at m/z 400, with the top 20 most abundant multiply charged (2+ and higher) ions In a given chromatographic window subjected to MS/MS fragmentation in the linear ion trap. An Fourier transform mass spectrometry (FTMS) target value of 1e6 and an ion trap MSn target value of 1e4 were used with the lock mass (445.120025) enabled. Maximum FTMS

scan accumulation time of 100 ms and maximum ion trap MSn scan accumulation time of 50 ms were used. Dynamic exclusion was enabled with a repeat duration of 45 s with an exclusion list of 500 and an exclusion duration of 30 s.

For mass-spectrometry data analysis, raw data files were processed using MaxQuant (Version 1.6.10.43) (Tyanova, Temu, and Cox 2016). Data were searched against a *Danio rerio* UniProt sequence database (downloaded July 2022) using the following search parameters: digestion set to Trypsin/P with a maximum of 2 missed cleavages, oxidation (M), N-terminal protein acetylation as variable modifications, cysteine carbamidomethylation as a fixed modification, match between runs enabled with a match time window of 0.7 min and a 20-min alignment time window, label-free quantification was enabled with a minimum ratio count of 2, minimum number of neighbours of 3 and an average number of neighbours of 6. A first search precursor tolerance of 20ppm and a main search precursor tolerance of 4.5 ppm was used for FTMS scans and a 0.5 Da tolerance for the scans in the scanning platform ITMS. A protein False discovery rate (FDR) of 0.01 and a peptide FDR of 0.01 were used for identification level cut-offs. Then, we proceeded to the statistical analysis.

Western Blot

Total protein concentration was determined using the micro BCA method (Micro BCA Protein Assay kit, ThermoFisher, Catalog # 23235). 15 µg of protein from synaptosome fractions were heated for 10 min at 70 °C at 800rpm in Novex Tricine SDS Sample Buffer. Proteins were resolved by electrophoresis on 12% SDS-PAGE gels using the Mini Gel Tank (Invitrogen, ThermoFisher Scientific, MA, USA). Subsequently, proteins were transferred to polyvinylidene fluoride membranes (PVDF) (Amersham Biosciences, Piscataway, NJ, USA) at 20 V for 1 hour at RT °C using the Mini Blot module (Invitrogen, ThermoFisher Scientific, MA, USA) and processed for immunoblot analysis. Membranes were blocked at RT for 1 h in blocking solution (0.2 M phosphate buffered saline pH 7.4 -PBS-, containing 5% non-fat dry milk -Cat. 1,706,404; Bio-Rad-, 0.1% Tween-20 -Sigma-Aldrich), followed by overnight incubation at 4 °C with purified Anti-NSF antibody (1:1000, Antibodies.com, Cat. No. A12714) diluted in blocking solution with 1% milk, Anti-STXBP1 antibody (1:1000 Antibodies.com, Cat. No. A12714) diluted in PBS containing 0.1% Tween-20, and Anti-GAPDH antibody (1:1000, Proteintech, Cat. No. 60004-1-Ig) diluted in blocking solution with 1% milk. After three washes (5 min each) at RT with PBS containing 0.1% Tween-20, blots were incubated for 1 h at RT with horseradish peroxidase conjugated rabbit anti-goat IgG (A5420; Sigma-Aldrich) or horseradish peroxidase conjugated donkey anti-rabbit IgG (NA934; Amersham Biosciences) secondary antibodies, all diluted to 1:10,000 5% milk.

After three additional washes, immunoreactive bands were visualized using an Odyssey CLx (LI-COR, Inc., Lincoln, NE). A colour pre-stained broad-range protein ladder (26616, ThermoFisher Scientific, MA, USA) was used to estimate the molecular mass of individual bands.

For the analysis, the membranes with only NIR ladder samples were scanned on an Odyssey CLx (LI-COR, Inc., Lincoln, NE) scanner at 700 and 800 nm. Fluorescent membrane scans were obtained using an Odyssey CLx controlled by Image Studio (LI-COR, Inc., Lincoln, NE). GAPDH was considered the loading control.

Experiment 1

Twenty-four hours after the end of the behavioural task, animals were euthanized by rapid cooling (~4ºC) and decapitated. Brains were dissected, flash frozen in liquid nitrogen and placed at – 80ºC to analyse for further synaptosome fraction extraction as address above.

Experiment 2

Some proteins of interest may have been altered by the anaesthetics, either during or after the animal was shocked, as this is the moment when the animal associated the black side with the aversive stimulus. Thus, in this experiment 2, the animals were only tested in the conditioning phase of the one trial inhibitory avoidance task, and 24 h after being exposed to the treatment, they were euthanized by rapid cooling (~4ºC) and decapitated. Brains were dissected and processed as referred in the experiment 1. Here, in addition to the control group (CN) that did not revisit the behavioural apparatus (post-conditioning phase), a control group (C) subjected to the full behavioural task was also tested. Thus, in addition to brain alterations caused by recall (experiment 1), here other altered proteins can be detected.

Statistical Analysis

Data was analysed using IBM SPSS™ 26 for Windows (SPSS Inc., Chicago, IL, USA) after exporting it to the Microsoft Excel™ 2010 (Microsoft Corporation, Redmond, WA, USA). First, data were analysed to verify normality (Shapiro-Wilk test) and homoscedasticity, using Levene's test.

Regarding behavioural results, data did not have a normal distribution, thus, Wilcoxon matched-pairs signed-rank test was use for comparisons between the same subject

regarding the latency to enter in the black side before and after the conditioning, and time spent in each side of the apparatus in the post-conditioning. Also, for comparisons between treatment groups, Kruskal-Wallis test with the post-hoc Dunn's test was used.

Regarding western blot results, as the data were normally distributed, one-way ANOVA with Tukey HSD post hoc test was used for comparisons between treatment groups.

Regarding MS data, protein group output files generated by MaxQuant were loaded into Perseus version 1.6.10.50. The matrix was filtered to remove all proteins that were potential contaminants, only identified by site and reverse sequences. The Label Free Quantification intensities were then transformed by log2(x), normalised by subtraction of the median value, and individual intensity columns were grouped by experiment. Proteins were filtered to keep only those with a minimum of 3 valid values in at least one group. The distribution of intensities was checked to ensure standard distribution for each replicate. Missing values were randomly imputed with a width of 0.3 and downshift of 1.8 from the standard deviation. To identify significant differences between groups, one-way ANOVA were performed with a permutation-based FDR of 0.05 followed by a Post hoc Tukey's HSD test with a permutation-based FDR of 0.05. The mass spectrometry data will be submitted to the ProteomeXchange Consortium via the PRIDE partner repository.

Results

Animals Removed from the Analysis

Six animals from the PL group, three from the MS222, and three from the control group were not considered for the behaviour analysis because they were outliers considering the time to enter the black side (median ±1.5 IQR). Furthermore, one animal from the MS222 group wasn't considered because it died during anaesthesia.

Behavioural changes

Animals in the propofol/lidocaine group do not remember the electric shock

The latency to enter the black side before and after conditioning is illustrated in Figure 4.2. Latency to enter the black side during the conditioning side was used to assess whether all groups behaved the same before being tested; no differences were observed between the groups. Regarding the comparisons before and after conditioning, the control and MS222 animals took longer to enter the black side after conditioning ($p= 0.011$ and $p= 0.003$, respectively), contrary to the P/L animals, which took a similar time. Additionally, P/L animals spent more time on the black side when compared to the time on the white side during post-conditioning ($p=0.002$); the other groups revealed no preference (Figure 4.3). Regarding the comparison between groups on the latency to cross to the black side on each testing phase, there were no differences between them.

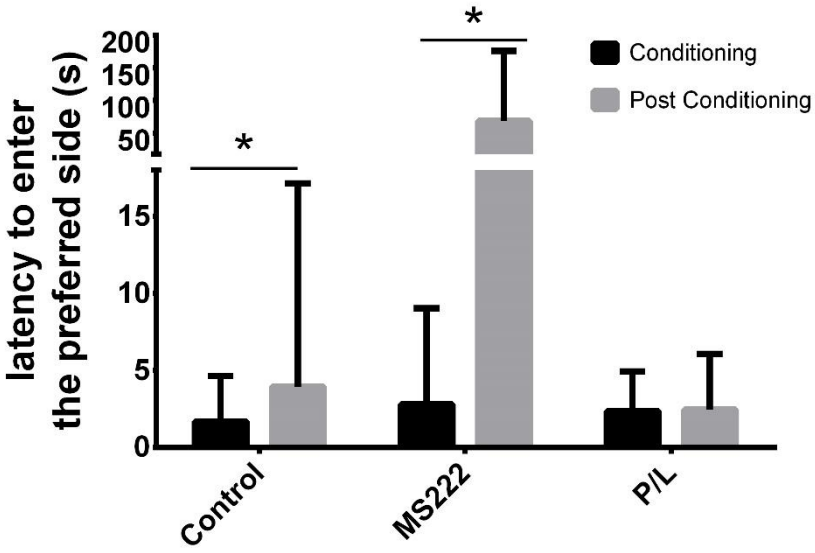

Figure 4.2: Latency to enter the conditioned side (black side) in the conditioning phase and in the post-conditioning phase for each treatment group in the one-trial inhibitory avoidance task; the black side was conditioned by an electric shock. Control group: animals subjected to clean water (n= 17); MS222 group: animals subjected to 150 mg/L of MS222 for 10 min (n= 17); P/L group: animals subjected to 5 mg/L of Propofol and 150 mg/L of Lidocaine for 10 min (n= 14). Data presented as median + IQR. * $p \leq 0.011$ for differences between phases within each group ($p \leq 0.03$) using Wilcoxon signed-rank test. To increase the visibility of the control and P/L group bars, we hide some values of the Y-axis due to the high latency values of the MS222 group.

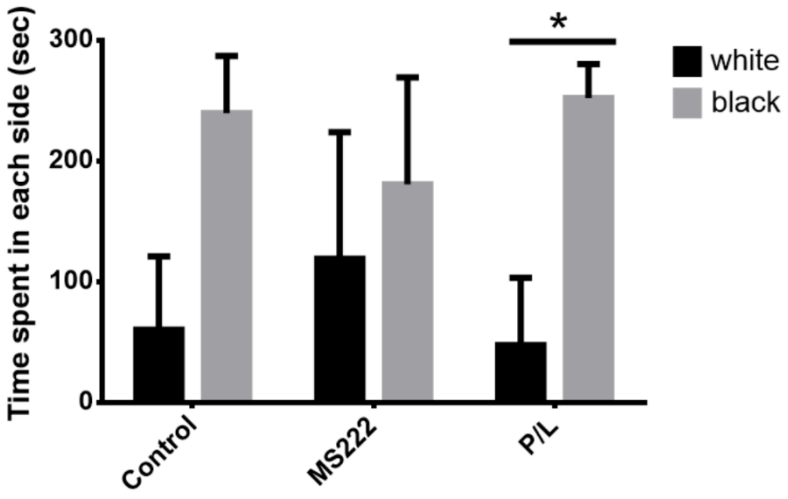

Figure 4.3: Time spent in each side in the post-conditioning phase for each treatment group in the one-trial inhibitory avoidance task in experiment 1; the black side was conditioned by an electric shock. Control group: animals subjected to clean water (n= 17); MS222 group: animals subjected to 150 mg/L of MS222 for 10 min (n= 17); P/L group: animals subjected to 5 mg/L of Propofol and 150 mg/L of Lidocaine for 10 min (n= 14). Data presented as median + IQR. * p = 0.02 for differences between phases in the P/L group using Wilcoxon signed-rank test.

Proteomic analysis of synaptosomes

For both experiments, for quality control and assessment of total coverage of the proteins identified, a comparison was made with the Bayes experiment [197] as this experiment has previously mapped the zebrafish synaptosome proteome. Here we could see that only 21 proteins were identified in experiment 1 that were not included in the Bayes experiment. And there was an overlap of 2600 proteins between the three experiments. The Bayes study shows more proteins, but we attributed that to differences in the synaptosome extraction and analysis. This data can be seen on the supplemental data annex (see annex 8).

Experiment 1: Analysis of the animals subjected to the full behavioural test.

Twenty-four hours after the end of the behavioural test, the synaptosomes of the animals selected for proteomic analysis were analysed as described in the methods section. From the 3221 proteins identified, 2075 met the parameters for being statistically analysed. 39 proteins had statistical differences between groups (permutation-based FDR 0.05), 13 of which were identified as associated with synapses and cognition (Table 4.1, blue cells). We used String (String, String consortium 2022, Switzerland) to create an interactome between

these 39 proteins (Figure 4.2); two were identified as of particular interest as they were associated with synapses and cognition in addition to having available antibodies for zebrafish. MS222, PL and Naïve presented statistical differences with the control group; the Naïve group exhibited a larger difference between medians followed by P/L and then MS222 for NSFb ($q<0.001$), while for STXBP1b only P/L and Naïve were different from the control group, with the Naïve group showing a larger difference between medians followed PL ($q < 0.04$). Thus, we performed western blotting to validate the different expression of NSFb and STXBP1b between groups, but no statistical differences were observed (Figure 4.3). Note that NSFb was the protein observed in the proteomic analysis, but NSF was the only antibody available for zebrafish; same as for STXBP1b, where the antibody purchased was for the STXBP1 protein, which although reactive with zebrafish, does not specifically marks STXBP1b, so both bands were present and overlapping in the western blot.

	M vs C	N vs C	P vs C	N vs M	P vs M	P vs N
adss2	-1.1	NS	NS	0.9	0.8	NS
gria3a	NS	NS	NS	1.3	1.1	NS
gstr	NS	-0.6	NS	-0.7	-0.3	0.4
nsfb	-4.7	-5.8	-5.5	NS	NS	NS
pcdh1a4	NS	NS	-1.2	NS	-1.1	-1.2
rab24	NS	NS	-2.2	NS	-1.5	-2.2
rab3aa	NS	-2.6	-2.4	-2.2	-2.1	NS
rac3a	NS	-1.8	-1.9	-3.4	-3.6	NS
rpl18	-1.5	NS	NS	1.6	1.6	NS
rpl39	NS	-2.3	NS	-2.0	NS	2.3
rps24	NS	-2.7	-2.0	NS	NS	NS
stxbp1b	NS	-6.0	-7.0	NS	NS	NS
usp9	NS	-1.6	-2.3	NS	NS	NS
atp5md	NS	6.3	NS	6.8	NS	-5.6
cdipt	NS	NS	-2.7	NS	-3.1	NS
clgn	0.4	0.4	0.4	NS	NS	NS
coro2ba	2.5	NS	NS	-2.5	-2.4	NS
dhrs4	NS	1.2	NS	NS	NS	-2.1
eps15l1a	-2.7	NS	NS	2.1	2.7	NS
fabp4a	NS	-0.7	-0.3	-0.3	NS	NS
gpm6aa	NS	-0.7	-1.0	NS	NS	NS
hbaa2	NS	-1.8	NS	-1.9	NS	2.7
hbba2	NS	-1.8	NS	NS	NS	2.4
ino80e	NS	-1.4	NS	-1.8	NS	1.9
LOC40287	NS	NS	-1.9	NS	-2.0	-2.2
mecr	NS	-1.9	NS	-2.8	-2.2	NS
mt-cyb	NS	3.2	NS	3.0	NS	-2.8
osbp	-0.9	-1.8	-2.1	NS	NS	NS
rgn	NS	-1.6	-2.9	NS	-2.1	NS
rpl7	NS	-0.5	-0.5	-0.6	-0.6	NS
rpl36	-3.4	-3.9	-4.4	NS	NS	NS
scrn3	NS	-0.8	NS	-0.4	NS	0.4
slc25a23a	NS	NS	NS	NS	2.4	2.7
sod1	NS	-0.4	NS	-0.4	NS	0.3
spcs3	NS	-3.0	-0.5	-3.0	NS	NS
syne1a	NS	NS	2.1	NS	2.5	NS
tpm3	NS	-0.6	-0.7	NS	NS	NS
tspan7b	-2.3	-2.4	-3.3	NS	NS	NS
zgc:12310	NS	-2.0	NS	-2.3	NS	NS

Table 4.1: Identification of statistically significant protein abundance changes between groups 24h after the one-trial inhibitory avoidance task. Proteins are represented with their gene names, except LOC402879, that still does not have a gene name identified; blue cells with gene names represent proteins associated with synaptic environments. Red or green shades mean the protein was significantly reduced or increased, respectively. The darker the colour, the larger the difference between groups. The pair of letters on the top of the table represents between each groups the differences were tested; M-MS222, C-Control; N- Naïve, P- Propofol/lidocaine.

Differences were calculated by comparing the median difference between protein groups using a one-way ANOVA followed by a Tukey HSD; NS- non-significant difference.

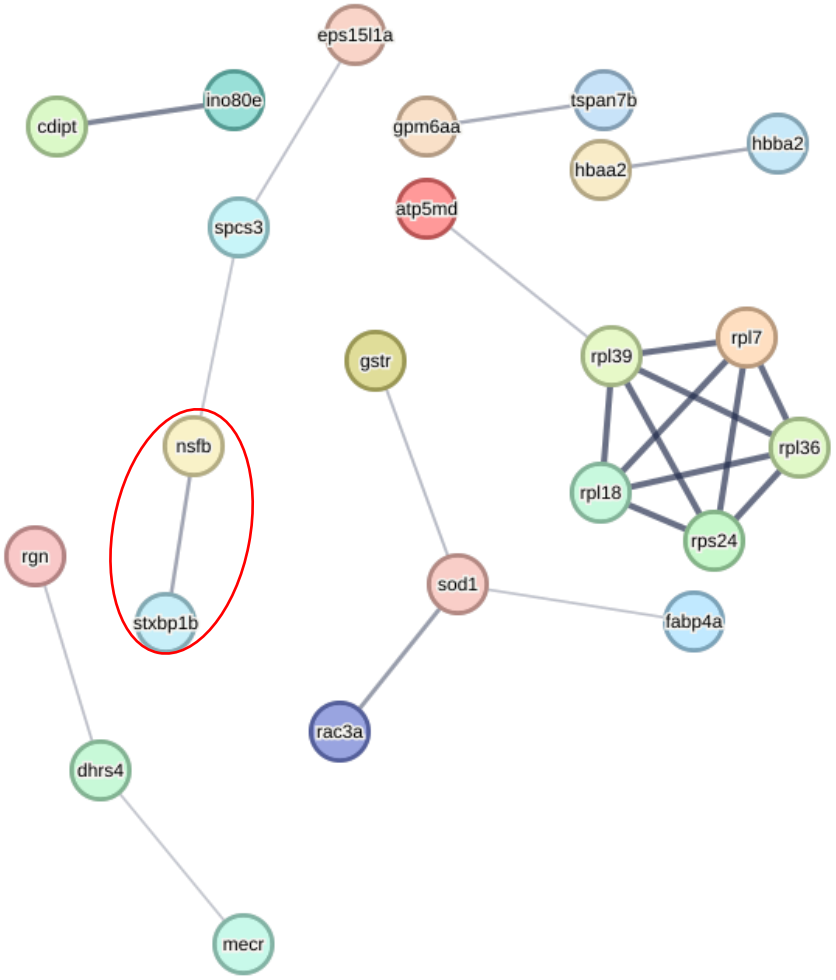

Figure 4.2: Protein-protein Interaction network of statistically significant proteins (different expression between groups) identified in the proteomic data. The red circle indicates two interacting proteins of interest identified as potentially being affected by our anaesthetic protocols and with available zebrafish reactive antibodies. This interactome was designed in String (String, String consortium 2022, Switzerland).

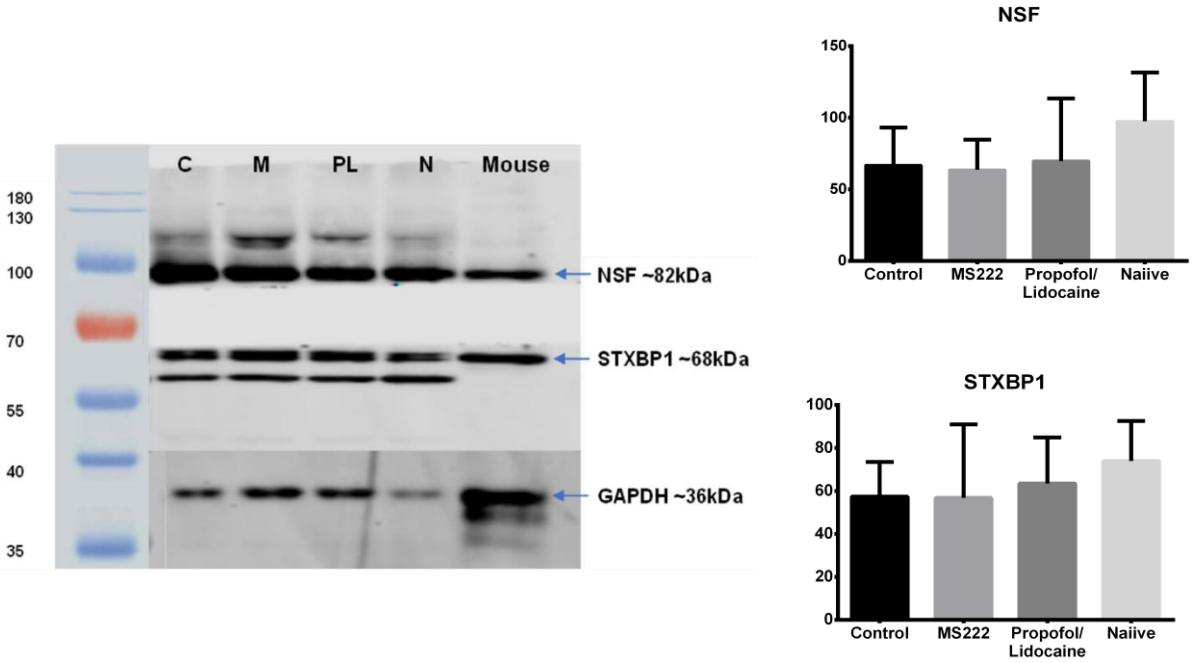

Figure 4.3: Western blot of NSF and STXBP1 in synaptosomes from the same groups analysed by mass spectrometry in experiment 1. The proteins analysed are indicated in our representative blots for each experimental group, and the mouse sample was a positive control for the antibodies; C: animals subjected to clean water; M group: animals subjected to 150 mg/L of MS222 for 10 minutes; P/L group: animals subjected to 5 mg/L of propofol and 150 mg/L of lidocaine for 10 min; N group- naïve animals that were not subjected to any treatment; all groups, except naïve, were tested in the one-trial inhibitory avoidance test. Bar graphs represent the median + IQR of the intensity of the bands normalized to the loading control GAPDH; n= 5. No significant differences were found using a one-way ANOVA with Tukey HSD post hoc test.

Experiment 2: Animals subjected to the conditioning phase of the behavioural task and anaesthetic exposure.

It is possible that revisiting the apparatus itself may elicit synaptic protein abundance changes, which could mask protein changes related to task learning/ acquisition. To address this possibility, we performed another experiment wherein the animals did not revisit the apparatus, i. e. no post-conditioning phase was tested. Thus, the animals were euthanised 24 h after anaesthetic or clean water exposure. Their brains were then dissected, and their synaptosome proteome was analysed as described for the previous task. Here, no overlap in significantly expressed proteins was found with the previous set of animals. From the 2747 proteins identified, 1731 met the parameters for being statistically analysed, and from these, 76 proteins had statistical differences between groups; 13 of them were associated with synapses and cognition (Table 4.2). Here, we used String [198] (String, String consortium 2022, Switzerland) to create a protein-protein interaction network

between these 76 proteins and two (SNAP25a and SNAP25b) were identified as proteins of interest (Figure 4) because they were associated with cognition and were also linked to both affected proteins from the experiment 1 (Figure 4.5). Unfortunately, there was no availability of antibodies for zebrafish for all the proteins with significant changes, so these proteins could not be immunoblotted to validate the proteomic data.

	CN vs C	CN vs M	CN vs P	M vs P
dnajc5aa	NS	NS	-0.5	NS
gphnb	NS	NS	-0.6	-0.6
gsk3ba	NS	NS	NS	NS
mdkb	NS	NS	-2.9	NS
ndrg4	NS	NS	-1.3	NS
nlgn3b	NS	NS	-2.6	NS
nrxn3a	NS	NS	-1.8	NS
rab5aa	NS	NS	-0.7	NS
slc12a5a	NS	NS	-0.4	NS
snap25a	NS	NS	-0.7	-0.3
snap25b	NS	-0.6	-0.7	NS
sncb	NS	NS	-1.2	NS
sncga	NS	NS	-2.3	NS
ahcyl1	NS	NS	-0.3	NS
anxa13	NS	NS	-1.0	NS
atp5f1c	NS	NS	-1.1	-0.7
atp6v0d1	NS	-0.5	-0.5	NS
atp6v1f	NS	NS	-1.7	NS
basp1	NS	NS	NS	NS
carmil2	NS	NS	-1.0	-1.3
cds2	-3.3	NS	NS	NS
cndp2	-1.9	NS	NS	NS
cyb5b	-1.5	NS	NS	NS
cyc	NS	NS	-4.2	-3.8
dhtkd1	NS	NS	-1.2	NS
dync1i2b	NS	NS	-1.3	-1.0
dynll2b	NS	-2.2	-2.5	NS
eif2s3	0.7	0.7	1.0	NS
ephb3a	NS	NS	-2.0	NS
epn1b	NS	NS	-2.1	NS
fdps	-6.2	NS	NS	NS
fech	NS	NS	-1.4	NS
gap43	NS	NS	NS	NS
gpam	-4.5	NS	-4.0	NS
histh1l1	NS	NS	1.4	NS
hnrnpa0l	-2.3	NS	NS	NS
hnrnpabb	NS	-2.6	-3.5	NS
hnrnpl2	NS	-1.8	NS	NS
hnrnpr	NS	-2.7	NS	NS
hprt1	NS	NS	-2.9	-2.5
isoc1	NS	NS	-1.8	NS
ldha	0.7	NS	NS	NS

lgals3a	NS	NS	-1.2	NS
marcksb	NS	-1.0	NS	NS
ndufa7	NS	NS	-4.3	-3.9
otub1b	NS	-0.9	-0.8	NS
palm1b	NS	-0.6	-0.8	NS
prdx5	NS	NS	-0.7	NS
prkacaa	NS	NS	NS	NS
ptgesl	NS	NS	-0.6	-0.4
ptmab	NS	NS	-1.8	NS
ptprfb	NS	NS	-1.8	-1.6
pvrl2l	NS	-0.5	-0.7	NS
rab14l	-2.3	NS	-2.2	-2.1
rpl19	NS	NS	2.4	NS
rpl22	NS	NS	1.8	NS
rpn2	-0.2	NS	NS	NS
s100b	NS	-1.7	-1.7	NS
scg2b	NS	NS	-0.7	NS
sdhc	-3.2	NS	NS	NS
si:dkey-276j7.1	NS	NS	-0.8	NS
si:dkey-56m19.5	NS	-1.1	NS	NS
slc2a1b	NS	NS	-2.2	NS
slc3a2b	NS	NS	-0.5	NS
srsf3b	NS	NS	-2.4	-2.2
srsf5a	NS	NS	NS	NS
stoml2	NS	NS	-1.0	-0.7
tpm1	NS	NS	NS	NS
tpp2	NS	-7.1	-7.0	NS
ttyh1	NS	-1.9	-2.0	NS
ttyh3b	NS	-0.7	-1.1	-0.3
ywhae1	NS	NS	-0.9	-0.4
ywhaqa	NS	NS	-0.8	NS
ywhaqb	NS	-0.6	-0.8	NS

Table 4.2: Identification of statistically significant protein abundance changes between groups in the experiment 2, where there was no post-conditioning phase. Proteins are represented with their gene names, except si:dkey-276j7.1 and si:dkey-56m19.5, which were not identified; blue cells with gene names represent proteins associated with synapses. Red or green shades mean that the protein was significantly increased or decreased, respectively. The darker the colour, the larger the difference between groups. The pair of letters on the top of the table represents between each groups the differences were tested; M- MS222, C- Control that revisited the apparatus; CN- Control that did not revisit the apparatus, P- Propofol/lidocaine. Differences were calculated by comparing the median difference between protein groups using a one-way ANOVA followed by a Tukey HSD; NS- non-significant difference.

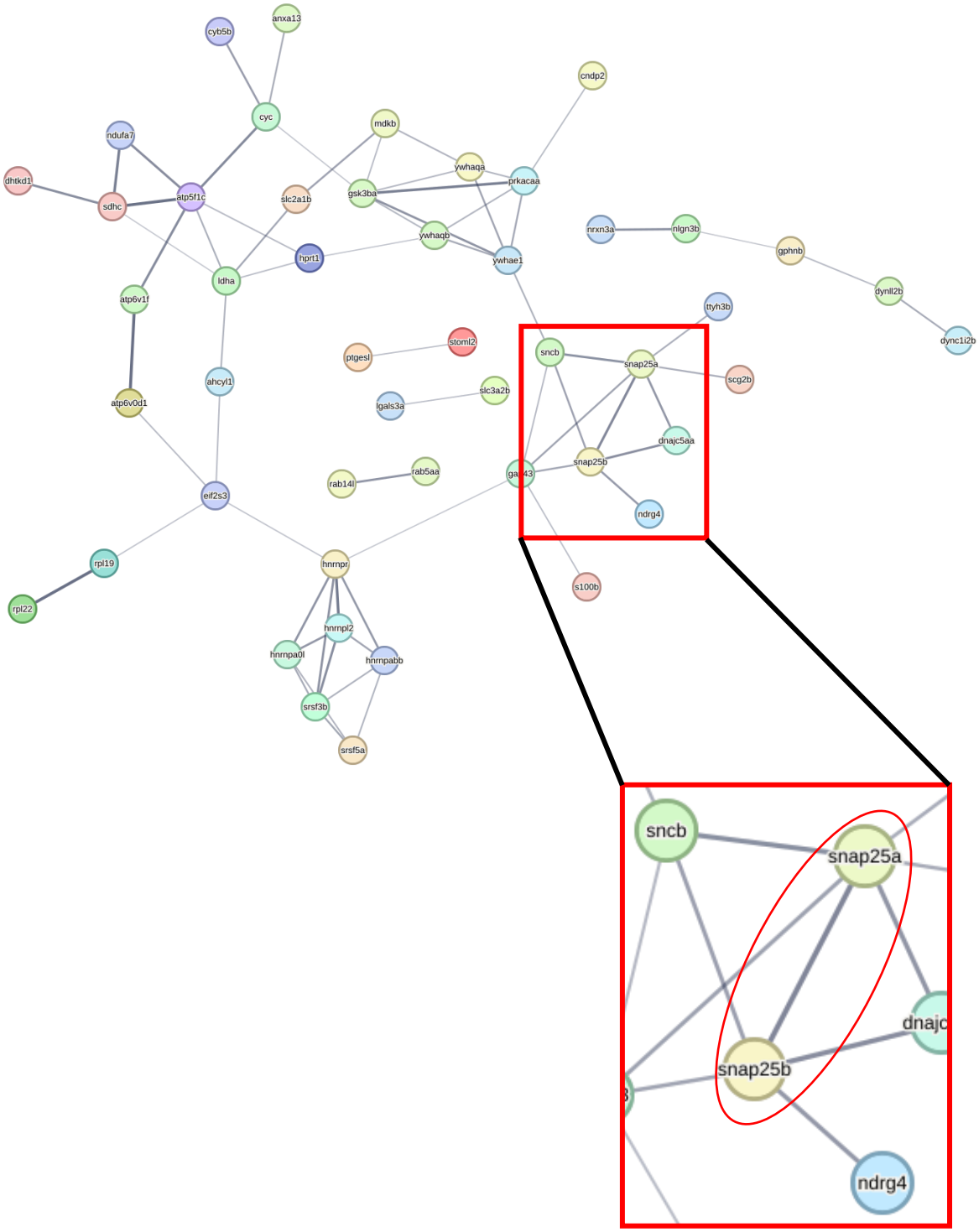

Figure 4.4: Protein-protein Interaction network of proteins expressed statistically different between groups in the proteomic data from task 2. This interactome was designed in String (String, String consortium 2022, Switzerland). The red square represents the area that is zoomed in the bottom. The red circle indicates two interacting proteins of interest that were identified as potentially being affected by our anaesthetic protocols.

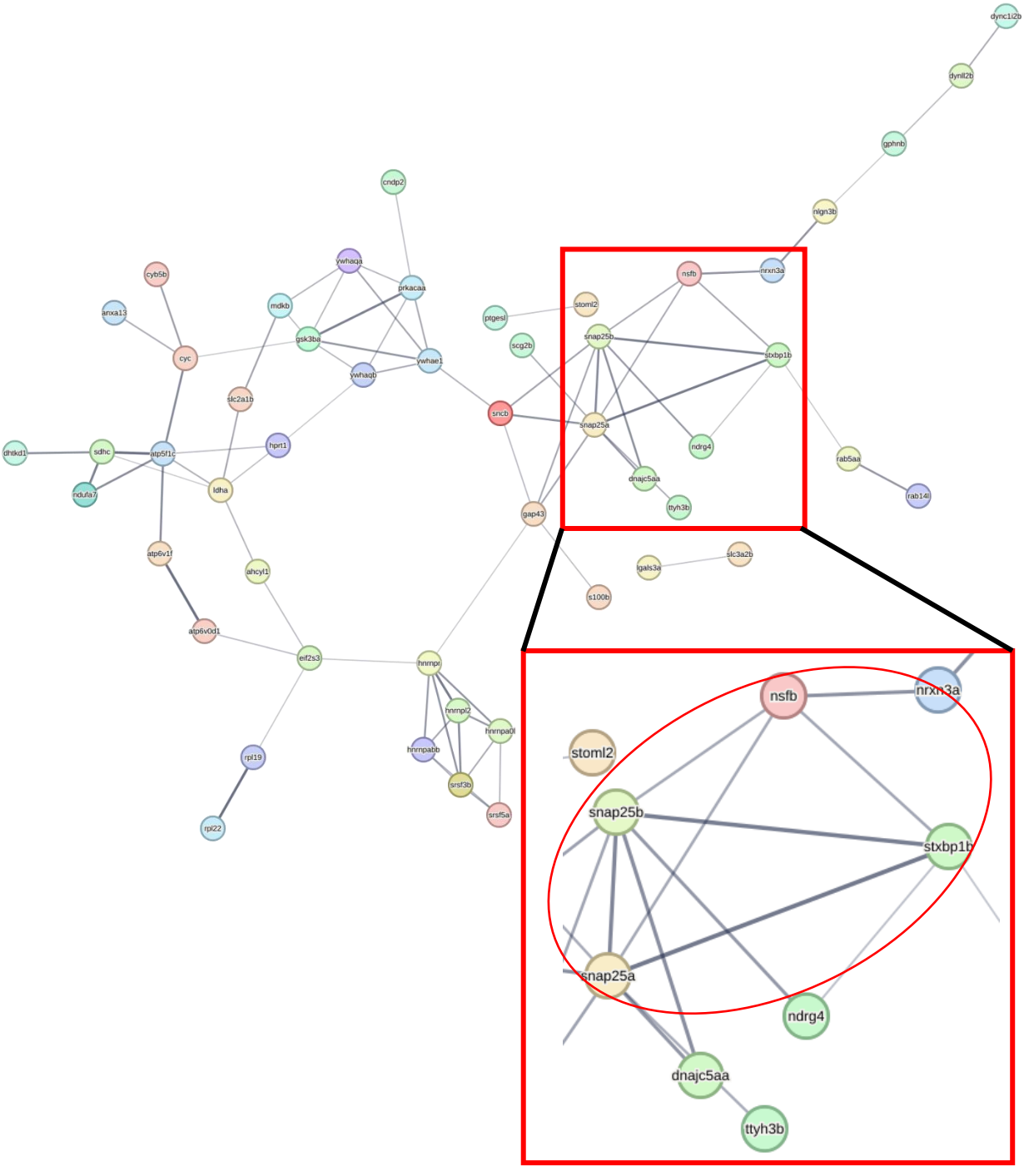

Figure 4.5: Protein-protein Interaction network of proteins expressed statistically different between groups in the proteomic data from experiment 1 and 2. This interactome was designed in String (String, String consortium 2022, Switzerland). The red square represents the area that is zoomed in the bottom. The red circle indicates the cluster of proteins of interest that were identified as potentially being affected by our anaesthetic protocols in both experiments that are also related to each other.

Discussion

Anaesthesia has been described to have the potential to induce long lasting changes on memory in humans and rodents [2, 4, 5, 22, 23, 32, 193, 194], but few studies have been performed with fish. As zebrafish is becoming extensively used as an animal model in neuroscience, this question become more relevant. Therefore, we intended to clarify if the standard anaesthetic MS222 or the promising combination propofol/ lidocaine (P/L) [65] induced changes on memory and how. The anaesthetic combination P/L caused memory impairment in a fear conditioning task, but not MS222. Nevertheless, both anaesthetic protocols induced alterations in the synaptosome proteins related to release of neurotransmitters and synaptic vesicle recycling.

We verified that for aversive learning, contrarily to the control group and MS222, animals subjected to a combination of propofol and lidocaine did not retain memory of the electric shock 24 h after exposure, showing a similar latency before and after conditioning. Also, the time P/L group spent in the black side during post-conditioning was higher than the time spent in the white side, while the other groups did not show that difference, indicating that the natural preference for the black side was conditioning by the shock. These results for the P/L group indicate an amnesic profile, as supported by our previous study validating this protocol with the amnesic MK-801 (Chapter 3).

To better understand what was happening at the molecular level, the synaptosome fraction of the animals was analysed by mass spectrometry to assess which proteins exhibited protein abundance changes in response to each anaesthesia protocol. Some proteins of interest related to synapse biology were identified as having a role on memory as well as having antibodies available that were reactive to zebrafish samples, such as the NSFb and STXBP1b; both proteins are related to exocytosis mechanisms in the synapse and the SNARE complex [199, 200]. In experiment 1, these proteins were more enriched in the control group compared to the naïve group, which did not have to learn the behavioural task, indicating the importance of these proteins for the memory process. Regarding the anaesthesia groups, STXBP1b and NSFb were reduced in the P/L group compared to the control group, while the MS222 group only revealed a lower abundance of NSFb. This might explain why MS222 presented no behavioural differences, contrary to the P/L group, which had two proteins affected that regulate memory formation [201, 202]. Thus, P/L may induce a decline in the exocytosis rate in synapses or recycling capabilities of the SNARE complex, which would explain the cognitive impairment observed. Unfortunately, this could not be validated through western blotting, and we attribute this to the fact that there is no available antibody for the fish specific b isoform for both NSF and STXBP1; we had to use commercial antibodies that probably recognise both isoforms as the peptide sequence was present in

both (information retrieved from UniProt [203]). As the mass spectrometry data showed no differences in isoform a, the differences related to b isoform in the western blot may be masked; it was technically impossible to differentiate both isoforms and quantify them alone because they have very similar molecular weights. Other proteins related to synaptic activity were involved in this behavioural task memory, as they were enriched in the control group compared with the naïve animals. From those, the Ras-related protein Rab-3Aa (RAB3aa), the 40S ribosomal protein S24, and the ubiquitin specific peptidase 9 were reduced in the P/L group compared to the control, supporting the deleterious effect of this anaesthetic protocol on this fear conditioning task. Again, the results of RAB3aa support the exocytosis and secretion of vesicles in the synapsis as one of the key processes affected [204].

We also wanted to know whether revisiting the apparatus (post-conditioning) could be sufficient to modify the expression of proteins by providing a new learning stimulus. In addition, we aimed to study if other proteins were affected during conditioning and several hours later, which may have returned to the baseline levels by 24h after post-conditioning and therefore were not observed as being different in experiment 1. Thus, experiment 2 was performed, and the proteins of the synaptosome of individuals that did not revisit the apparatus after conditioning was analysed using mass spectrometry 24h after anaesthesia exposure. Here, we could see no overlap of significantly altered proteins when comparing both experiments, leading us to hypothesize that acquiring/consolidating and recalling a memory is differently impacted by the anaesthetics. The results show similar levels of synaptic proteins between control and CN, which points to the proteins assessed being equally important for both consolidation and recall. An interesting finding was that SNAP25a and SNAP25b were affected in this experiment. These proteins are related to synaptic vesicle docking and are implicated in synaptic plasticity [205]. SNAP25b was depleted in both anaesthetic groups, while SNAP25a was only affected by P/L. With these proteins being connected to the proteins of the first experiment (Figure 4.5) (data from STRING interactome database [198]), this can give us more clues to justify the difference of memory alterations between MS222 and P/L group.

Other proteins related to synaptic activity were involved in this behavioural task memory in experiment 2. P/L alone significantly reduced multiple proteins also associated with vesicle trafficking (dnajc5aa, nlgn3b, nrxn3a, rab5aa, and sncga). This supports the deleterious effect results of P/L as it gives further information on how it might affect synaptic communication.

Summing, from our data, we propose that 24h after anaesthesia exposure, when the memory of the task may still be consolidating, there may be a deficient formation of the SNARE complex; the use of MS222 depleted one of the units, while the P/L decreased two

of the SNARE units. Then, revisiting the apparatus (post-conditioning), MS222 reduces NSFb, which is insufficient to prevent neuronal communication, as animals exposed to MS222 can still recall the shock memory. However, the depletion of two components of the SNARE complex induced by the P/L may lead to problems in the acquisition/ consolidation of the shock memory, with the added difficulty that recall may also be impaired by the alteration of synaptic vesicle docking and fusion, and SNARE recycling via STXBP1b and NSFb, respectively [201, 202, 205].

Thus, these results support the behavioural observation that P/L and not MS222 impaired the inhibitory avoidance task. Nevertheless, another study showed that MS222 impaired working memory and cognitive flexibility until two days after anaesthesia exposure [6]. Therefore, the influence of anaesthesia on memory depends not only on the type of anaesthetic protocol but also on the type of memory studied. It is known that MS222 induces cognitive impairment in more complex tasks [206], which are different from the one studied here as it is a fear-based aversion task. This fear-based task can produce a stronger memory as it is closely linked to survival, which might be the reason why we cannot see the decline in our MS222 animals.

This work shows that anaesthesia alters memory-related proteins differently depending on the anaesthetic protocol. It also sheds light on how anaesthesia might impact different processes of memory formation, especially by affecting proteins involved in exocytosis and recycling of synaptic vesicles. MS222 seems less deleterious than the P/L protocol. From another perspective, P/L could be useful for the animals to forget stressful situations, improving their welfare.

Concluding, these findings suggest that there might be a deficient formation of the SNARE complex during the first 24 hours after anaesthesia exposure that leads to problems in the acquisition/consolidation of memory, with recall being possibly impaired by alterations in synaptic vesicle docking and fusion, and SNARE recycling via STXBP1b and NSFb, respectively. Overall, this study provides valuable insights into the effects of anaesthetics on memory formation and the underlying molecular changes that occur in zebrafish.

General discussion

Although the anaesthetic process is effective, it is a process that is still not fully understood [192]. There are still risks not considered/mitigated, such as aversion to the anaesthetic and/or cognitive impairment. With cognitive impairment impacting so many individuals and with the possibility to be impacting many more not being accounted, this work aims to get more information. Such information will span across the mechanisms of anaesthesia which are of general interest and can provide more information to mitigate those side effects in the future. At the same time, these studies aim to provide a comprehensive analysis of anaesthesia for the animal model zebrafish, by evaluating the commonly used anaesthetic and possible alternatives focusing on a holistic approach to assess the impact of these on animal welfare and research outcomes.

Anaesthetic protocols should be selected based on their efficacy and reduced side effects, considering animal welfare compliance and scientifically sound data. The perception of drugs as aversive by animals can affect their behaviour and physiology, inducing distress and reducing welfare. MS222, the most used anaesthetic for zebrafish, has been reported to be aversive [7, 10], leading to the search for better alternatives. We then decided to compare the behavioural and physiological effects of MS222, propofol/lidocaine, clove oil, and etomidate on zebrafish. While all the tested protocols were not innocuous, animals treated with MS222, propofol/lidocaine, and clove oil showed different behavioural profiles when compared to a positive control for aversion (HCl), contrary to etomidate. Additionally, from a physiological point of view, all anaesthetic treatments, except etomidate, presented similar cortisol levels. Etomidate blocks cortisol release, which can also influence the experiments.

Although our results differ from those of Readman et al. [7], where animals exposed to etomidate did not show aversion and animals exposed to MS222 did show aversion signs, the differences can be attributed to the different paradigms used in the studies, ours involved conditioned learning, and a higher concentration of anaesthetic was used to mimic an experimental situation where animals had to be anaesthetized and may have felt aversion. Additional different results were seen [9] when attempting to replicate the experiment conducted by Wong et al. [10], which reported that animals treated with MS222 showed aversion. However, in the present study, animals treated with MS222 did not present a clear aversion profile, especially when comparing the same variable as Wong, the time spent in each side of the tank. The differences may be due to the testing of animals in pairs in the Wong et al. study [10], which may have influenced the results due to shoaling or territorial behaviours, and the absence of environmental enrichment, unlike in the present study where animals were tested individually and had an enriched environment. Our group

tested the paradigm with pairs before [9] with only MS222 and P/L, but the results were not clear and no aversion was also registered. There, animals treated with P/L took longer to enter the compartment where the anaesthetics were presented when compared to the training baseline, which may we hypothesize being due to the white colour of propofol [9]. However, in the present study, animals treated with propofol/lidocaine and etomidate showed no differences in latency to enter the conditioning side comparing the exposure phase with the post-conditioning phase, when the animal was exposed to the white-coloured Intralipid vehicle, ruling out the possibility of visual cues skewing the results.

Overall, the results of this study suggest that MS222, propofol/lidocaine, and clove oil may be less aversive to zebrafish than previously thought, and etomidate may be more aversive than reported in previous studies. Clove oil was considered a less desirable candidate because of its smell and need to be previously prepared by adding ethanol. So, with this in mind, the matter of MS222 and P/L being amnesic was still in place, and for that we need a test that was easy to use, fast to learn, generating a long-lasting memory and allowed to assess memory performance. Such test chosen was the one-trial inhibitory avoidance task [134] and we decided to create our own behavioural apparatus with accessibility and cost in mind.

Accessibility and cost are also crucial components that allow others to replicate experiments, and this promotes the standardization of methodologies. Unfortunately, there is a methodology report crisis because methods are often not fully described, which leads to difficulties in replicating experiments [168]. We encountered this and needed to validate an electric shock that produced enough aversion for the animals to avoid the context where they were shocked. Zebrafish strains are known to prefer the black compartment in the apparatus used in these tasks, possibly due to the hazards associated with the white colour and its increased visibility to predators [207]. After conditioning, the goal is for the animal to learn to avoid the black side by learning that such context might mean another shock. In this study, electrical parameters were tested, and it was found that a combination of 3V and 2A for 5 seconds produced consistent conditioning. The animals required more time to travel to the black side, indicating that they found the shock unpleasant. The apparatus was then validated as it creates an aversive response and has the potential to be used to study the effects of drugs on memory and learning capabilities, as well as to assess other parameters and preferences such as colour and patterns.

We then focused on the inhibitory avoidance task in zebrafish, which assesses learning by testing the ability of the animals to remember and avoid an electric shock. To validate that the task is evaluating what we want (memory), we used the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 as a positive control, which has been shown to induce memory

impairment in this type of task [180]. The results of the study showed that the MK-801 animals did not remember the shock, as they took the same time to go to the black side before and after the shock. In summary, we have validated an inhibitory avoidance task in zebrafish using a newly designed apparatus with refined parameters for the electrical shock. Our results showed that the shock induced aversion in the animals and the NMDA antagonist MK-801 disrupted memory formation in both zebrafish strains tested. Overall, this validated inhibitory avoidance task can be used as a tool to study learning and memory processes in zebrafish to evaluate the effects of drugs on these processes.

With the validated test, we went and checked if and how the anaesthetics assessed in Chapter 1 and chosen as potential candidates affect memory. The study aimed to investigate whether the standard anaesthetic MS222 and the combination propofol/lidocaine (P/L) induced changes in memory and synaptic activity in zebrafish. The results showed that the P/L combination caused memory impairment in a fear conditioning task, but not MS222. However, both anaesthetic protocols induced alterations in synaptosome proteins related to the release of neurotransmitters and synaptic vesicle recycling. Mass spectrometry analysis of the synaptosome fraction showed that the decrease of specific proteins, such as NSFb and STXBP1b, which are involved in exocytosis mechanisms and the SNARE complex, may explain the cognitive impairment observed in the P/L group. On the other hand, the use of MS222 decreased only one unit of the SNARE complex, which was not enough to prevent animals from recalling the shock memory. Further, revisiting the apparatus showed that the decrease in NSFb and SNAP25b was not enough to prevent neuronal communication, and animals exposed to MS222 could still recall the shock memory. However, the decrease in two components of the SNARE complex induced by the P/L may lead to problems in the acquisition/consolidation of the shock memory, with the added difficulty that recall may also be impaired by the alteration of synaptic vesicle docking and fusion, and SNARE recycling via STXBP1b and NSFb, respectively. Overall, this study highlights the importance of investigating the effects of anaesthesia on memory formation and synaptic activity in zebrafish as it is becoming an extensively used animal model in neuroscience.

This thesis highlights the importance of considering aversion when selecting anaesthetic protocols to ensure animal welfare compliance and provision of scientifically sound data, as distress may influence animals' behaviour and physiology. Additionally, this study contributes to the already extensive work showing the importance of quality reporting of research [168]. Providing details of methodology enables efficient replication of previous studies, reducing additional work by other research groups; our study is an example of that, as we add to perform extra work to study the adequate properties of the electrical shock to induce the minimal aversion to induce memory formation in the behavioural task. Our

studies also show that all anaesthetics in a way impact zebrafish. Being it through behavioural aversion, impact on cortisol synthesis, memory/learning, and brain proteins. Additionally, it also seems like MS222, contrarily to what have been reported, does seem to be an adequate anaesthetic for a daily use on our facilities as far as aversion to our anaesthesia concentration is concerned. It also shows that P/L is a possible alternative that introduces a smaller workload, although keeping in mind that its impact on our studies needs to be taken into account as it seems to modulate memory, at least 24h after anaesthesia. Nevertheless, it may be useful to decrease the distress of a stressful event, as, if P/L is given after that event, it may induce amnesia of that experience.

Finally, this shows that there is an important effect of both anaesthetics, similar to what happens in humans from a behavioural perspective, on major synaptic exocytosis related proteins which by itself opens the door for further research to try and clarify the role of anaesthesia on POCD and create a further strategy to counteract these effects.

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Supplemental material

Annex 1 – Supplementary table for CPA

	Exposure	Post-exposure
Group	Solution (colour)	Solution (colour)
Clove Oil	Clove Oil (clear)	Water (clear)
Propofol/Lidocaine	Propofol/Lidocaine (white)	Intralipid (white)
Etomidate	Etomidate (white)	Intralipid (white)
Vehicle	Intralipid (white)	Intralipid (white)
MS222	MS222 (clear)	Water (clear)
HCl	HCl (clear)	Water (clear)

Supplemental table 1: Description of the chemical added to the water in the Exposure and Post-exposure phase and respective colour of the solution.

Annex 2 – Supplementary data for CPA

ID	Treatment	Days to learn	Entrance in the lighted side	Number of entries in the lighted side	Phase
1	MS222	3	1	1	Post-exposure
2	MS222	7	1	1	Post-exposure
3	MS222	5	1	10	Post-exposure
4	MS222	4	1	18	Post-exposure
5	MS222	7	1	1	Post-exposure
6	MS222	7	1	1	Post-exposure
7	MS222	9	1	9	Post-exposure
8	MS222	5	1	4	Post-exposure
9	MS222	5	1	5	Post-exposure
10	MS222	10	1	12	Post-exposure
11	MS222	6	1	1	Post-exposure
12	MS222	7	1	30	Post-exposure
13	Vehicle	6	1	1	Post-exposure
14	Vehicle	3	1	26	Post-exposure
15	Vehicle	6	1	1	Post-exposure
16	Vehicle	7	1	11	Post-exposure
17	Vehicle	4	1	9	Post-exposure
18	Vehicle	10	1	7	Post-exposure
19	Vehicle	6	1	3	Post-exposure
20	Vehicle	4	1	16	Post-exposure
21	Vehicle	7	1	9	Post-exposure
22	Vehicle	6	1	20	Post-exposure
23	Vehicle	10	1	2	Post-exposure
24	Vehicle	10	1	11	Post-exposure
25	Clove Oil	5	1	1	Post-exposure
26	Clove Oil	8	1	2	Post-exposure
27	Clove Oil	6	1	1	Post-exposure
28	Clove Oil	4	1	1	Post-exposure
29	Clove Oil	7	0	0	Post-exposure
30	Clove Oil	9	1	1	Post-exposure
31	Clove Oil	5	1	13	Post-exposure
32	Clove Oil	6	1	11	Post-exposure
33	Clove Oil	4	1	13	Post-exposure
34	Clove Oil	3	1	7	Post-exposure
35	Clove Oil	3	1	33	Post-exposure
36	Clove Oil	3	1	28	Post-exposure
37	HCL	4	0	0	Post-exposure
38	HCL	6	0	0	Post-exposure
39	HCL	5	1	4	Post-exposure
40	HCL	7	0	0	Post-exposure
41	HCL	3	0	0	Post-exposure
42	HCL	4	1	1	Post-exposure
43	HCL	4	1	2	Post-exposure
44	HCL	8	0	0	Post-exposure
45	HCL	6	1	1	Post-exposure
46	HCL	9	0	0	Post-exposure
47	HCL	3	0	0	Post-exposure
48	Propofol/Lidocaine	4	1	10	Post-exposure
49	Propofol/Lidocaine	6	1	8	Post-exposure
50	Propofol/Lidocaine	4	1	6	Post-exposure
51	Propofol/Lidocaine	6	1	2	Post-exposure
52	Propofol/Lidocaine	4	1	1	Post-exposure
53	Propofol/Lidocaine	5	1	24	Post-exposure
54	Propofol/Lidocaine	10	1	4	Post-exposure
55	Propofol/Lidocaine	10	1	8	Post-exposure
56	Propofol/Lidocaine	5	1	7	Post-exposure
57	Propofol/Lidocaine	10	1	27	Post-exposure
58	Etomidate	7	1	5	Post-exposure
59	Etomidate	7	1	3	Post-exposure
60	Etomidate	6	1	1	Post-exposure
61	Etomidate	5	1	6	Post-exposure
62	Etomidate	4	1	11	Post-exposure
63	Etomidate	3	1	1	Post-exposure
64	Etomidate	4	0	0	Post-exposure
65	Etomidate	4	0	0	Post-exposure
66	Etomidate	6	0	0	Post-exposure

Supplemental table 2: Compilation of the raw data for each treatment group regarding days to achieve the learning criteria, binary result of entering the lighted side, and the number of entries in the lighted side during the post-exposure phase. side and the number of entries in the lighted side during the post-exposure phase.

Annex 3 – Code file

```
// C++ code

int buttonState = 0;

int counter;


void setup()

{

  pinMode(13, OUTPUT); // Internal led

  pinMode(8, INPUT);   // Button for zap

  pinMode(5, OUTPUT);  // On LED

  pinMode(4, OUTPUT);  // Test LED

  pinMode(7, OUTPUT);  // Relay

}


void loop()

{

  digitalWrite(5, HIGH);


  buttonState = digitalRead(8); // Check if button was pressed or not

  if (buttonState == HIGH) {    // If button is pressed

    delay(10);


   for (counter = 0; counter < 5; ++counter) { // Starts to switch the relay on and off the number of times in the counter (change value after < to change number of cycles of shock)

      // Turn LED on and close relay circuit in a NO relay component

      digitalWrite(7, HIGH);

      digitalWrite(4, HIGH);

      digitalWrite(13, HIGH);

      delay(1000); // Change value to increase time of shock

      // Turn LED off and open relay circuit in a NO relay component

      digitalWrite(7, LOW);

      digitalWrite(4, LOW);

      digitalWrite(13, LOW);
```

```
      delay(500); // Change value to increase time of pause between shocks
    }
  }
  else {  // If not pressed or exceeding the number of times in the counter
    // Turn the LED off and open relay circuit in a NO relay component
    digitalWrite(4, LOW);
    digitalWrite(7, LOW);
    digitalWrite(13, LOW);
  }

  delay(10); // Delay a little bit to improve simulation performance
}
```

Annex 4 – Circuit File

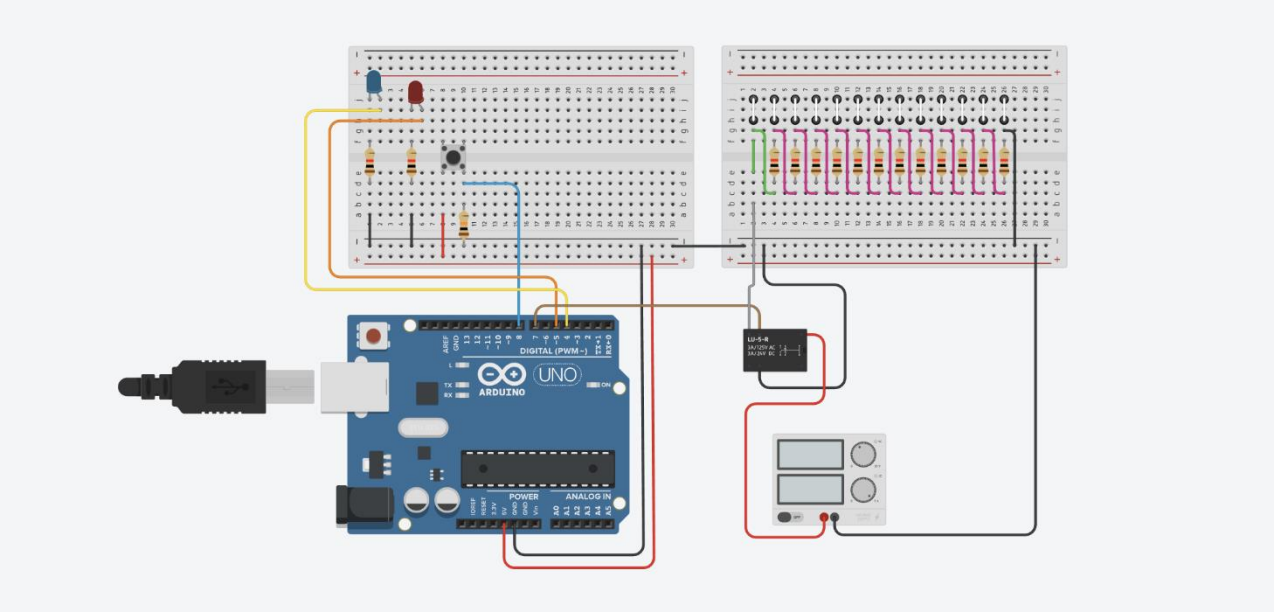
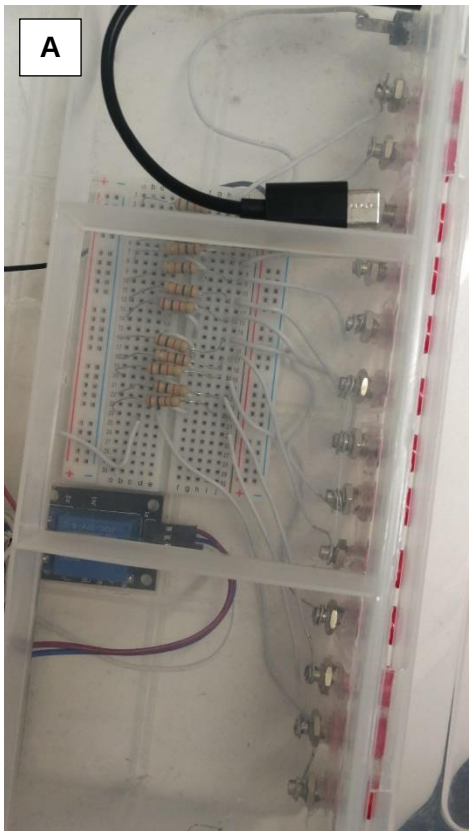
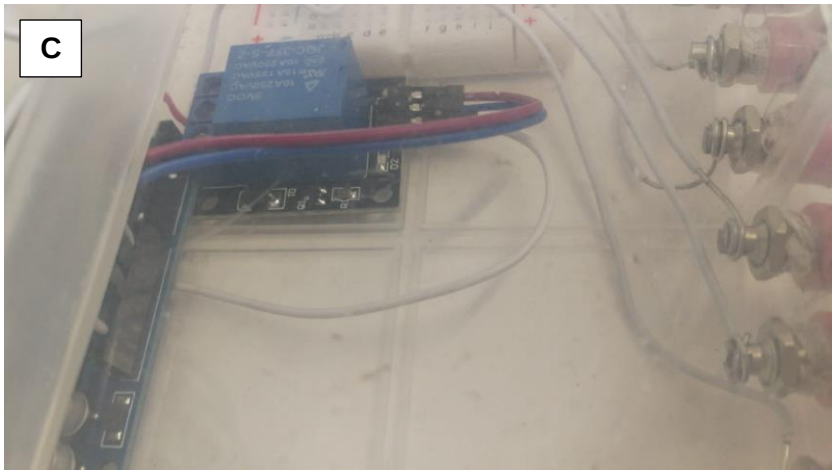
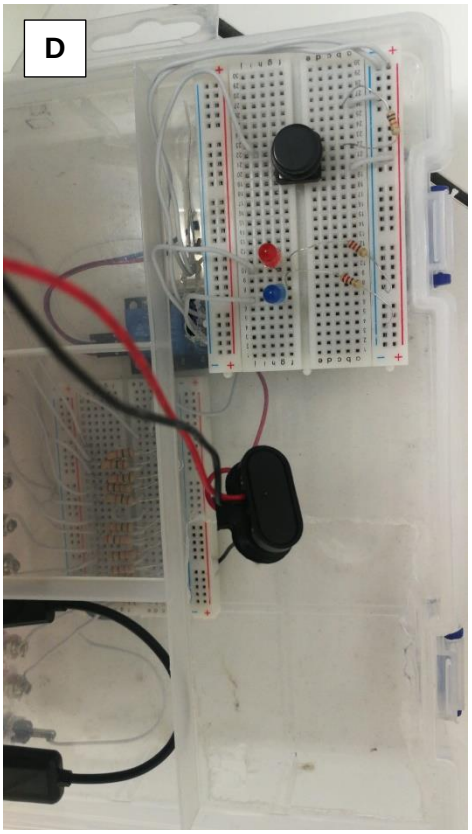


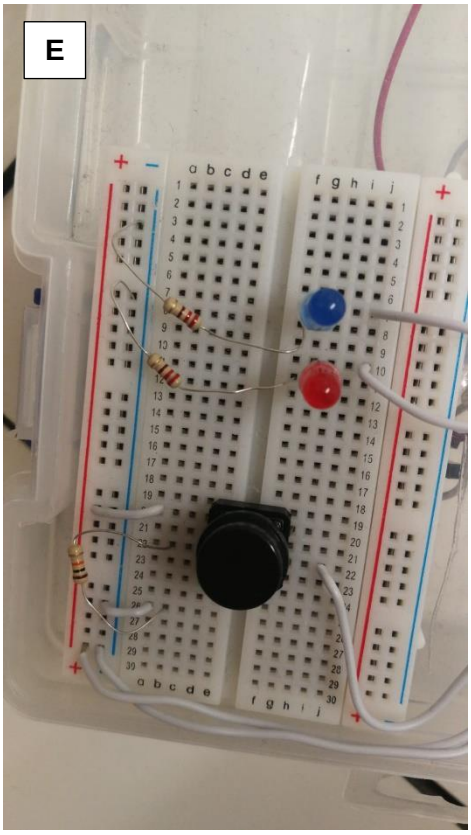
Figure S1: Schematic of the circuit layout of the shock unit with the voltage divider add-on.

Annex 5 – Building visual aid

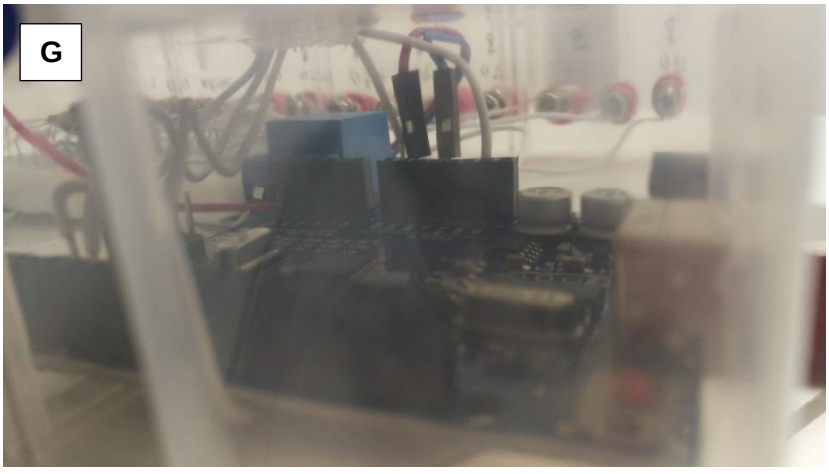





D


E


F


G

A – Top view voltage divider

B – Close up out plugs

C – Relay view

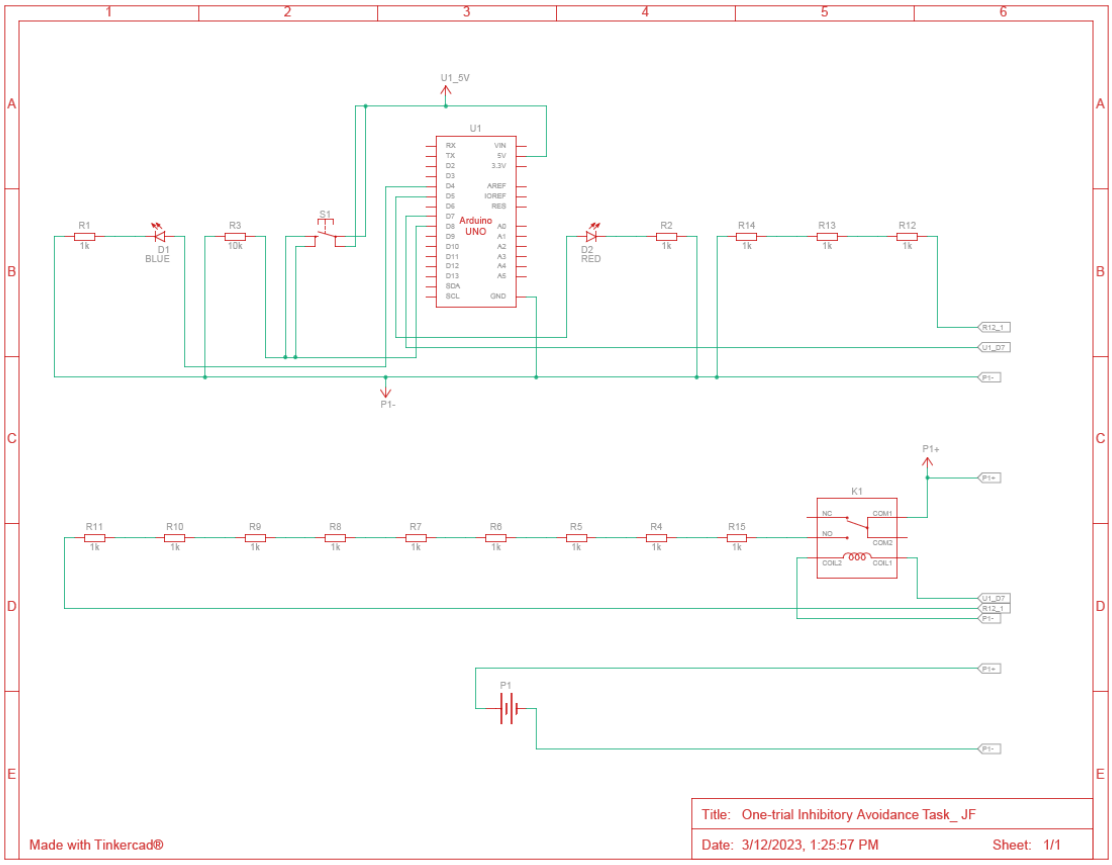
D – Top view main breadboard

E – Close up main breadboard

F – Close up plugs

G – Close up arduino

Annex 6 – Circuit layout



Name	Quantity	Component
U1	1	Arduino Uno R3
S1	1	Pushbutton
D1	1	Blue LED
D2	1	Red LED
R1, R2, R4, R5, R6, R7, R8, R9, R10, R11, R12, R13, R14, R15	14	1 kΩ Resistor
R3	1	10 kΩ Resistor
K1	1	Relay SPDT
P1	1	Power Supply

Annex 7 - Does anaesthesia affect classical learning-associated proteins?

Behaviourally it was possible to measure the predicted effects in the experiment 1 that exposed animals to anaesthetics, thus the classical learning-associated proteins were the next step to be assessed to check if the behaviour observed could be translated into a molecular component. This step was mainly done as a pilot before doing the Mass spectrometry analysis as we wanted to be sure that there were alterations in proteins classically linked to learning/cognition. Additionally, we also wanted to be sure that the extraction protocol was working and that we could see a clear difference in the quantity of synaptic proteins between the supernatant and the synaptosome fraction.

We decided to assess if synaptophysin (Abcam ab32594 1:5000 1% milk TBST), PSD-95 (NovusBiological NB300-294, 1:500 TBST), CaM Kinase II alpha (CAMKII) [phosp Tyr286] (NovusBiological NB300-184 1:1000 1% Milk TBST), BDNF (NovusBiological NB100-98682 1:1000 TBST), GABA-AR α 2 (NovusBiological NBP2-36560 1:1000 1% Milk TBST), NMDAR2B (phospho Tyr1472) (NovusBiological NB300-182 1:1000 1% Milk TBST) [208-211] had altered levels assessed by western blot with GAPDH (Genetex GTX100118 1:5000 1% Milk TBST) as the loading control. Although the antibodies available for this task were either reactive or predicted to react with zebrafish, only synaptophysin and CAMKII produced a signal that was possible to quantify. In the figure S3, we can see a clear difference in the quantity of synaptophysin in the synaptosome fraction. These results showed us, that the protocol of synaptosome fraction extraction was working as synaptophysin is only expressed in the synapse and the quantity in the supernant was very scarce. With these results, both proteins were less present on the propofol/lidocaine group when compared to the control group, which correlates with the behaviour data previously mentioned (figure S4).

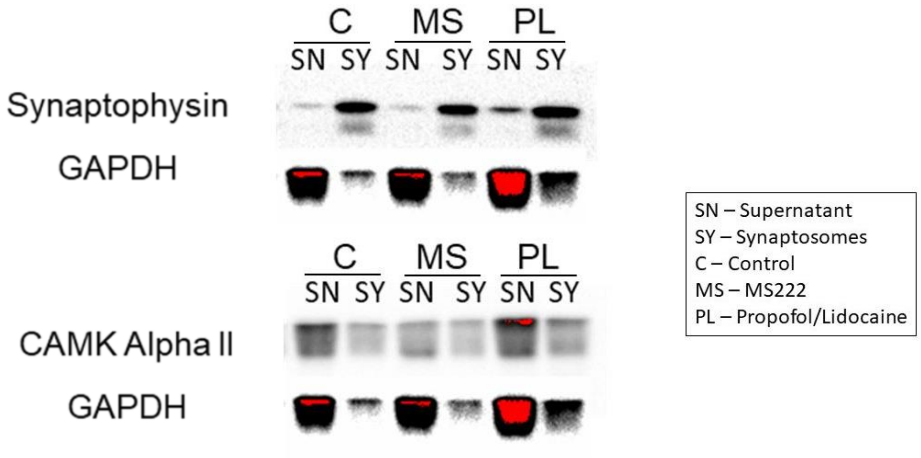


Figure S3: Western blot of the synaptosomes and supernatant collected from adult zebrafish immediately after the post-conditioning of the one-trial inhibitory avoidance task to ensure the quality of the synaptosome fraction separation.

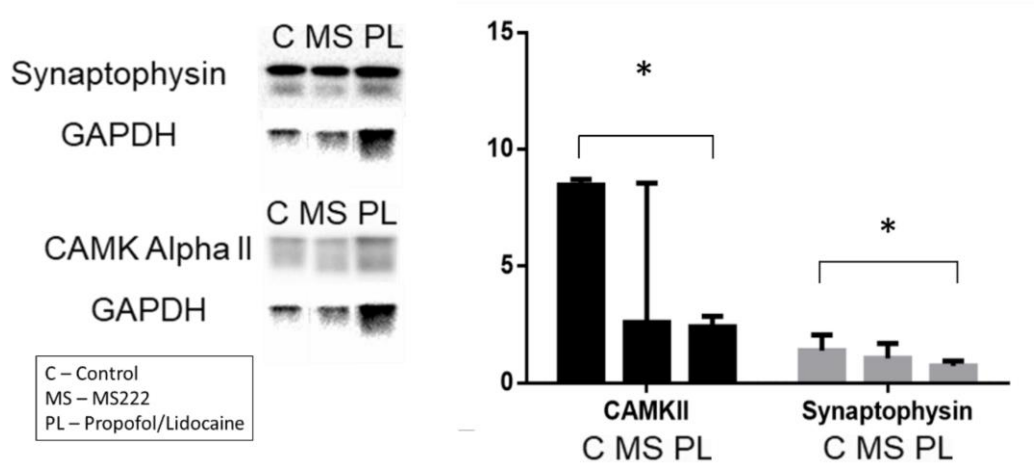


Figure S4: Western blot of the synaptosomes collected from adult zebrafish immediately after the post-conditioning of the one-trial inhibitory avoidance task. n= 4, 3, 5 for control, MS222 and propofol/ lidocaine, respectively, for protein quantification; *: differences between control and propofol/lidocaine anaesthetic protocol regarding synaptophysin and CAMK Alpha II, using Kruskal-Wallis test ($p<0.05$).

However, we could not find differences for these proteins on our Mass Spectrometry data. Either the differences were quite small and not picked up by Mass spectrometry, or we had a false positive in our western blot results, although, the behavioural differences correlate to the results obtained.

Annex 8 - Degree of coverage of our Mass spectrometry data

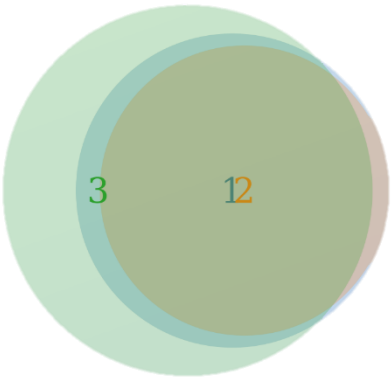


Figure S5: Overlap of proteins identified on both experimental datasets and the proteins identified by Bayes et al. 1 is the experiment 1 (in blue), 2 is the experiment 2 (in orange), and 3 is Bayes dataset (in green), where zebrafish neuroproteome was characterized. Number of total proteins: 1 – 3221; 2 – 2747; 3– 4486. Proteins only found on each experiment: 1 – 21, 2 – 0, 3 -1402. Number of common proteins in different datasets: overlap only between 1 and 2 – 116, overlap only between 1 and 3 – 453, overlap only between 2 and 3 – 0, overlap between all the datasets – 2631.

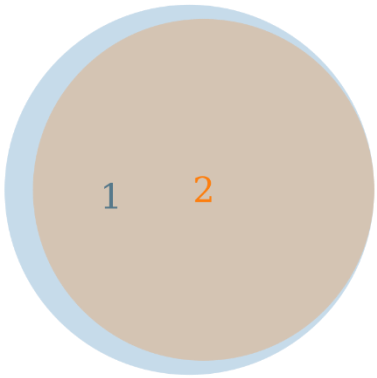


Figure S6: Overlap between both data sets of our experiments. 1 is experiment 1 (in blue), 2 is the experiment 2 (in orange). Number of total proteins: 1 – 3221; 2 – 2747. Proteins exclusive to 1- 474; Proteins exclusive to 2 – 0. Number of common proteins: 1 and 2 – 2747.

Annex 9 – Protein trend data

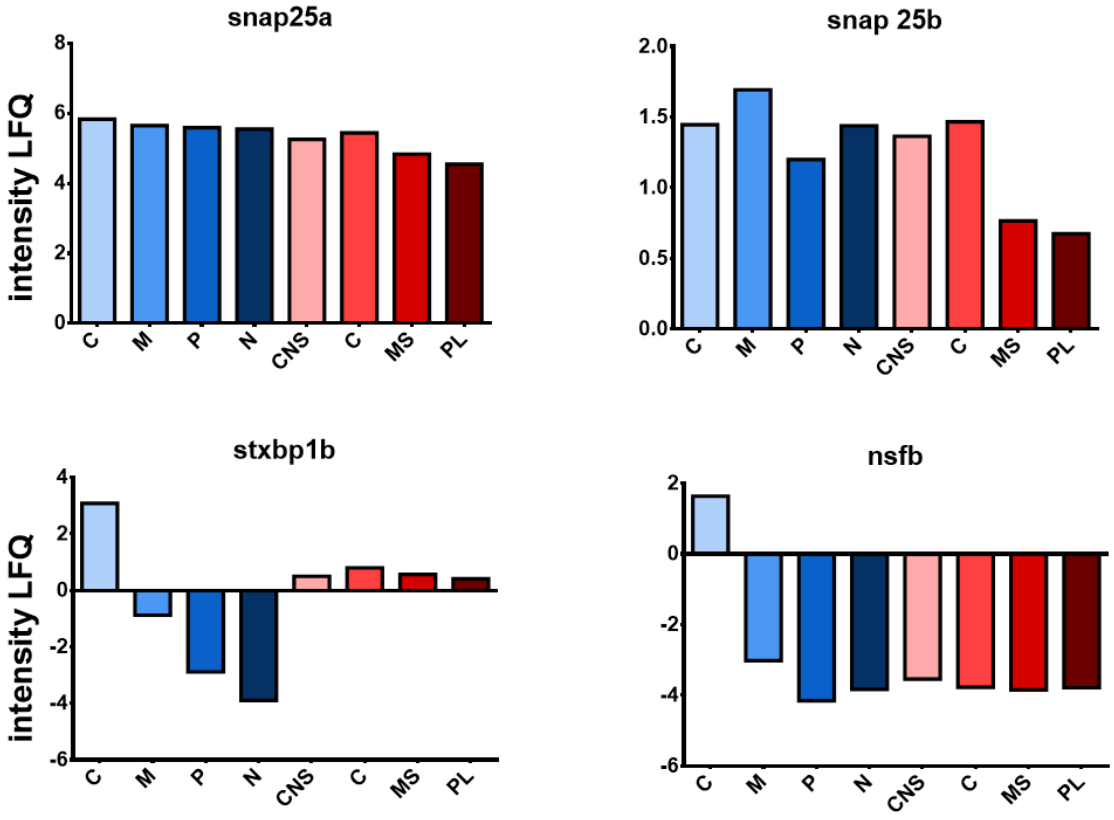


Figure S7: Average intensity LFQ for each protein in each group analysed. Bars of blue shades represent experiment 1. For experiment 1, C – Control, M – MS222, P – Propofol/Lidocaine, N – Naïve. Bars of red shade represent experiment 2. For experiment 2, CNS – Control without revisiting the apparatus, C – Control revisiting the apparatus, MS – MS222 without revisiting the apparatus, PL – Propofol/Lidocaine without revisiting the apparatus.