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Zebrafish anesthesia and potential implications in research:

Ketamine anesthesia and its influence in the development of Zebrafish central nervous system

Maria Margarida Martins Neves

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Dissertação apresentada à Universidade de Coimbrapara cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação da Professora Doutora Anália Carmo e da Professora Doutora Emília Duarte (Universidade de Coimbra)

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A tiny fish, no wider than your thumbnail,

may someday make a big difference to your health,

but not because it’s going to show up on your dinner plate.

FDA

Abreviations

5HT5-hydroxytryptamine receptors

AIF Apoptosis Inducing Factor

AMPA-R α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acidreceptor

BMP Bone Morphogenetic Protein

BrdUBromodeoxyuridine

cGMP cyclic Guanosine-mono-phosphate

CNS Central Nervous System

FDA Food and Drug Administration

GABAGamma-AminoButyric Acid

GFAP Glial Fibrillary Acidic Protein

HCN1 Hyperpolarisation-activated Cyclic Nucleotide channels

LC3 microtubule-associated protein 1A/1B-light chain 3

MS222 Tricaine Methanesulfunate

mTOR Mammalian Target of Rapamycin

nACh-R NicotinicAcetylcholineReceptors

NMDA-R N-methyl-D-aspartatereceptor;

NONitric Oxide

NPC Neural Percursor Cells

NSC Neural Stem Cells

PCNA Proliferation Cell Nuclear Antigen

PCP Phencyclidine

VDCC Voltage Dependent Calcium Channel

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Abstract

The use of Zebrafish as a model organism in scientific laboratories has increased in the past years. It has many advantages: bridges the gap between cellular models and complexes animal models, it is less expensive, easy to handle and generates large offspring. Additionally has a rapid development that occurs ex vivo which accompanied by its transparency allow complete, noninvasive developmental studies. Moreover, the use of Zebrafish raises less ethical questions, and its outcomes can be safely extrapolated to humans, due to high homology. However, investigation regarding its welfare and refinement techniques has not been much explored. The unknowing of side effects of regular procedures such as anesthesia can introduce an undesired variable into the studies, hence compromising result interpretation. Clarifying the secondary effects of anesthetics and finding a safe, adequate alternative to current ones is a necessity in zebrafish research.

Ketamine is a widely used anesthetic in veterinary and pediatrics, and therefore it could constitute an option in Zebrafish research. Nevertheless, the results regarding its effects in neurodevelopment are contradictory and its safeness is not clear. A correct understanding of the cellular pathways induced by this anesthetic, in the central nervous system is needed in order to improve animal wellbeing and to decrease variability in results obtained.

Considering that currently there is not a completely safe anesthetic and that refinement of anesthesia is a need in health sciences research, the present study aims to assess the cellular alterations induced by ketamine in the central nervous system of Zebrafish during development. Zebrafish were exposed to different concentrations of ketamine at different stages of development and were then collected and analyzed at different periods of time. Upon collection, animals were used to evaluate cell proliferation by two different methods: BrdU incorporation and Proliferation Cell Nuclear Antigen (PCNA) detection using immunohistochemistry and western blot. In order to study apoptosis, the DNA degradation, was assessed, the expression of caspase-3 and the expression of the apoptosis inducing factor (AIF) were evaluated by western blot and immunohistochemistry. To study autophagy, the expression of microtubule-associated protein 1A/1B-light chain 3 was evaluated by western blot and immunohistochemistry.

The results suggested that none of the ketamine concentrations altered the cell proliferation. This effect seemed to be independent of the embryo development stage. The study of the PCNA expression by immunohistochemistry also showed that the cell proliferation was restricted to specific areas such as: dorsal telencephalic proliferation zone, tectal proliferation zone and mesencephalic lamina in the mesencephalon, and cerebellum. Due to several technical difficulties it was not possible to accomplish the study of apoptosis nor of autophagy. Considering the results regarding cell proliferation, and the importance of ketamine in anesthesia it is important, in a near future, to evaluate the molecular mechanism associated to the maintenance of the cell proliferation, namely the role of neural stem cells. In addition, it is important to clarify the occurrence of

apoptosis and autophagy and its role in the maintenance of the cellular proliferation and/or remodeling of the CNS upon the exposition to ketamine.

Key words: Zebrafish, Anesthesia, Ketamine, Central Nervous System Development, Proliferation

Resumo

O uso do peixe Zebra (Daniorerio) como organismo modelo em ciência aumentou significativamente na última década, devido às vantagens que acarreta: complete os estudos celulares e é menos complexo que outros modelos animais, como os roedores. É barato, reproduz-se frequentemente e em grande número, é fácil de manipular, desenvolve rápida e externamente, o que juntando à sua transparência permite um estudo detalhado e não invasivo do seu desenvolvimento. Levanta menos questões éticas e, devido à elevada homologia com o Humano, os seus resultados podem ser extrapolados. No entanto, investigação direcionada para o bem-estar animal e refinamento de técnicas não foi muito abordada. O desconhecimento dos efeitos secundários de procedimentos rotineiros, tal como anestesia, pode introduzir uma variável indesejável nos estudos, comprometendo a interpretação dos resultados. Assim, a compreensão dos efeitos secundários dos anestésicos e a procura de uma alternativa adequada, é uma necessidade corrente na investigação em peixe zebra.

A cetamina é um anestésico comumente usado na medicina veterinária e em pediatria e constitui uma alternativa à anestesia atualmente usada. Não obstante, os seus efeitos no neurodesenvolvimento são contraditórios e a sua segurança não é clara. Uma compreensão do mecanismo celular em resposta aos anestésicos no sistema nervoso central é necessária para melhorar o bem-estar animal e também diminuir a variabilidade introduzida nas experiências.

Tendo em conta a falta de segurança da anestesia usada em peixe zebra e a necessidade de refinamento da técnica, o presente estudo tem como objetivo estudar as alterações celulares resultantes da administração de cetamina no sistema nervoso central deste ciprinídeo, durante o seu desenvolvimento. Uma administração pontual e uma exposição contínua (24h) foram testadas, em três concentrações diferentes, em três estádios de desenvolvimento do peixe zebra. Os grupos tratados foram recolhidos a diferentes horas, e vários parâmetros foram analisados. A proliferação celular foi avaliada por dois métodos: a expressão de PCNA por westernblot e imunohistoquímica e a incorporação de BrdU através de um ensaio colorimétrico. Ambos os ensaios revelaram não haver diferenças significativas na proloferação celular nos grupos tratados com cetamina, em comparação ao grupo controlo. Apoptose, autofagia, pluripotência e organização do citoesqueleto foram igualmente avaliadas mas, devido a dificuldade técnicas, não foram obtidos resultados. Assim, apesar de os resultados sugerirem que a cetamina não afeta a proliferação celular, mais estudos para avaliar a apoptose e diferenciação celular em resposta à cetamina são necessários para entender o papel do anestésico no desenvolvimento do sistema nervoso central e a sua potencial segurança no uso em peixe zebra.

Keywords: Zebrafish, Anestesia, cetamina, desenvolvimento do Sistema nervoso central, Proliferação

1.Introduction

1.1.Zebrafish: a novel model organism

Although mammal models are very valuable in health science research, its use is very controversial among the scientific community not only for ethical reasons that regularly arise, but also because the results are questioned. In fact, the concern with the wellbeing of animal models such as mice, rats and guinea pigs, led to the imposition of strict rules with regard to the maintenance, reproduction and research use of these animals. In addition, recent studies showed that results obtained with these animals are influenced by environmental variables and also by the interaction between the researcher and the animal. Moreover, the experiments are frequently limited by the number of available animals because offspring is not as abundant as one expected and it is expensive to obtain and maintain certain strains. Considering the limitations of the mammal models, some studies used, as an alternative model, the cellular lineages. However, those lack the microenvironment interactions seen *in vivo* and that are essential to understand most of the physiologic and pathological processes. (Hunter, 2008; Festing and Wilkinson, 2007; Rollin, 2007) Considering all limitations, for a decade, several researchers started using other animal models, among which is the Zebrafish. (Spitsbergen and Kent, 2003)

Danio rerio, commonly known as Zebrafish, is a thumbnail sized aquatic vertebrate that rapidly became one of the most used animal model in health science research. In 2012, it was the second most used organism, after mice, in scientific experiments from several areas. (ASPA, 2013; Fleming,2007) Zebrafish started to be used as a model for developmental studies and gene function. Later, zebrafish was used as a model for human neurodegenerative diseases including Parkinson's, Huntington's, and Alzheimer's as well as a model for cancerogenesis, inflammatory processes, and metabolic diseases. (Langenheinrich, 2003; Kalueff*et al.,* 2014). In addition, Zebrafish is considered a model for developmental toxicity and for that reason it has been used to screen chemical libraries in pre-clinical studies, to determine the mode-of-action of several drugs as well as to identify their toxic and teratogenic effects.

The success of zebrafish as a model in research is due to several reasons. Zebrafish is a vertebrate and thus closer to humans than invertebrate models, it is easier to manipulate and maintain, is less expensive than other animal models and raise less ethical issues than vertebrate models. Moreover, its transparency and *ex utero* development turn zebrafish an adequate model to study embryogenesis and organogenesis. (Fleming, 2007; Esche*et al.,*2012; Howe *et al.,* 2013). Zebrafish undergo a rapid development: the body plan is laid out in 24 hours post-fertilization (hpf) and they achieve an adult-like stage in 72hpf hence reducing the time of each experiment when compared to mice. Together, the transparency and the rapid development, allow the study of morphology, developmental stages of all organs, including the nervous system development. Morphologic and internal abnormalities are thus easily observed under an optic microscope without the need of invasive techniques, when compared to mammal models. Mutagenesis, the use of reporter genes and fluorescent markers are also possible and

combined with the genetic tools available are a huge advantaged in research with Zebrafish. (Goldsmith, 2004; Kalueff*et al.,*2014)

Zebrafish also reproduces periodically and generates a large offspring (100-200 embryos per breeding of a single pair of adults), which guarantees a constant availability of experimental subjects. In addition, their size accounts as another important advantage: the small size allows an easy manipulation in multi-well plates. Moreover, since Zebrafish can be maintained in as little as 50µl of fluid, a considerate amount of chemicals is saved when compared to mammal models. Zebrafish is also a good model for the *in vivo* drug screening since the administration of drugs is easy: hydrophilic molecules or small sized could be added directly to the water, passing to the fish system by diffusion or orally; hydrophobic or larger molecules could be injected in the yolk sac or *sinus* (Goldsmith, 2004).

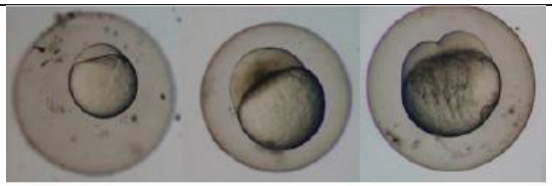
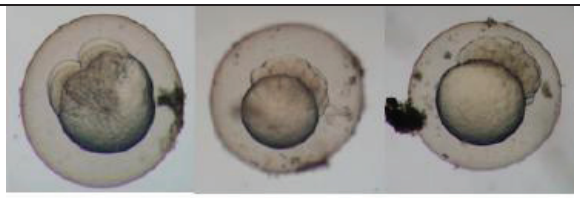
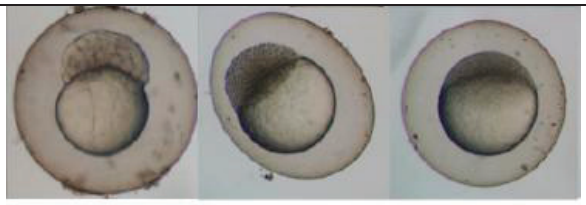
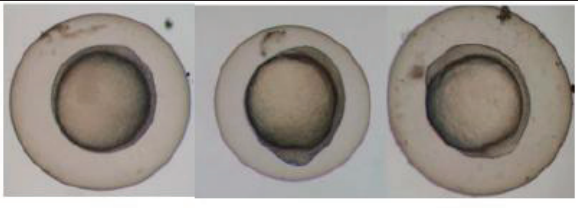
Together, these observations make Zebrafish a time/ cost-effective alternative to mice and other mammal models, allowing to overcome the gap between *in vitro* and *in vivo* work. But the virtues of Zebrafish as an animal model do not stop here: its genome is well described and most of it was already sequenced offering a high genetic tractability. Approximately 70% of its genes have human orthologs, of which about 80% are disease related genes. Amino-acid sequences of important protein domains are also proven to be evolutionary conserved. (Reimer*set al.,* 2004; Ali *et al.,*2011) This proximity of Zebrafish with humans and other higher vertebrates suggests similar cellular and molecular signaling pathways, supporting the validity of the experiments and the use of zebrafish as an animal model to study human pathologies. In fact, most of Zebrafish mutant phenotypes are similar to the phenotypes observed in several human disorders allowing an accurate analysis of several human pathologies and the genetic background associated. (Lewis, 2003; Moens and prince, 2002; Dooley and Zon, 2000). Embryological processes and development pathways are also conserved among zebrafish and mammalian. Nonetheless, if zebrafish embryonic neurotoxic responses are predictive of human ones, is still to be clarified.

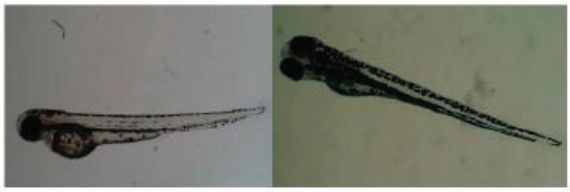
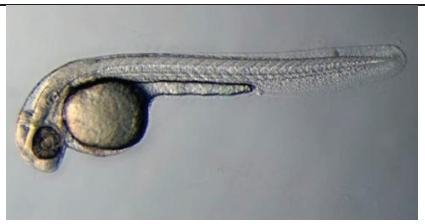
All the characteristics of Zebrafish make it an ideal, easy and reliable model, especially in integrated approaches that include exposure of an organism to a compound during different development stages. According to current European legislation this model organism is considered as an alternative compatible with the 3R's: Refinement, Reduction and Replacement of the use of rodents and other models in anim*al* experiments. (directive 86/609/EEC). However, there are certain aspects of its handling that are not very clear, namely the establishment of an adequate anesthetic method that allows the maintenance of the well-being and that does not interfere with the results of the experiment.

1.2.Zebrafish development

Zebrafish's development is divided in periods, defined by Kimmel. (Kimmel *et al.,* 1995) Embryogenesis (first three days after fertilization) has 5 broad periods: zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching, as summarized in table 1. After the third day, Zebrafish are called larvae, by this time they begin to swim, to escape from predators and to feed. The description provided for each period aims to highlight what is known about morphogenesis and main occurrences that take place during each period.

Table 1: Stages of embryonic development of zebrafish (Kimmel *et al.,*1995). Images are from: embryology.med.unsw.edu.au (consulted in 18th May,2015).

Period	Hours	Description	Photo
Zygote	0 - 0.75 hrs	Fertilization activates cytoplasm movements, and the latter accumulates at the animal pole. First cell division.	 zygote 1-cell 2-cell
Cleavage	0.75 - 2.25 hrs	Comprises 6 cell cycles, thatoccur synchronously.	 4-cell 32-cell 64-cell
Blastula	2.25 - 5.25 hrs	Mid Blastula Transition. Cell cycles are now asynchronous; Formation of Yolk syncytial layer and epiboly begins.	 128-cell 2K-cell oblong
Gastrula	5.25 - 10.33 hrs	Morphogenetic movements lead to formation of primary germ cell layers and embryonic axis. Epiboly is complete and tail bud is formed.	 80% epiboly tailbud 2-somite

Segmentation	10.33 - 24 hrs	Somites development, primary organogenesis and embryo elongation. First body movements.	 5-somite 18-somite 20-somite
Pharyngula	24 - 48 hrs	Body axis straighten, heart begins to beat and circulation, pigmentation, and fins begin to develop.	 prim-5 prim-11
Hatching	48-72 hrs	Morphogenesis of primary organ systems is almost complete and cartilage starts to develop in head and pectoral fin; the hatching occurs asynchronously during the whole period.	 48-hour 72-hour
Larvae	72 h	Morphogenesis complete. Swimming, jaw and eye movement begins. Heart-beat is now regular; yolk extension almost empty.	

One particular aspect in zebrafish development it is the presence of the corion until the hatching period is reached. The corionis porous (pores diameter is around 0.5–0.7 µm, and spaced 1.5–2.5 µm interval) and functions as a barrier to slow down diffusion of molecules from the water to the embryo, even for hydrophilic substances.(Dahm and Geisler, 2006; Braunbeck*et al.,* 2005; Braunbeck and Lamme, 2006; Lee *et al.,* 2007) For this reason, studies performed before the hatching period should be carefully planned to guarantee that the molecules can pass through the corion and are delivered to the embryo in an adequate concentration. In some studies it was developed a methodology to remove the corion in order to overcome this difficulty. (Ali *et al.,* 2011)

1.3.Zebrafish Central Nervous System Development

Central Nervous System (CNS) of Zebrafish arise from ectoderm and mesoderm interaction. It starts forming around 6hpf, during gastrulation, when the cells that will originate neurons and glial cells move to certain positions in Zebrafish embryo and begin the regional patterning (Kimmel *et al.,* 1990; Woo and Fraser, 1995). Similarly to other vertebrates, a layer of ectodermal epithelium on the dorsal side of the embryo, the neural plate is the primordial CNS structure. After several differentiation and rearrangement processes (around 9-10hpf) the neural plate leads to the neural tube formation, in which cells divide at the apical side and differentiate at the basal one. Afterwards the neural tube undergoes morphogenesis and is subdivided in different regions: forebrain (diencephalon and telencephalon), midbrain, hindbrain and spinal cord. At 24hpf this division is evident (figure 1), but ventricles formation and stimuli responses are only seen at 48hpf. (Wilson *et al.,* 1990; Eisen, 1991; Haffter *et al.,* 1996; Kawai *et al.,* 2001)

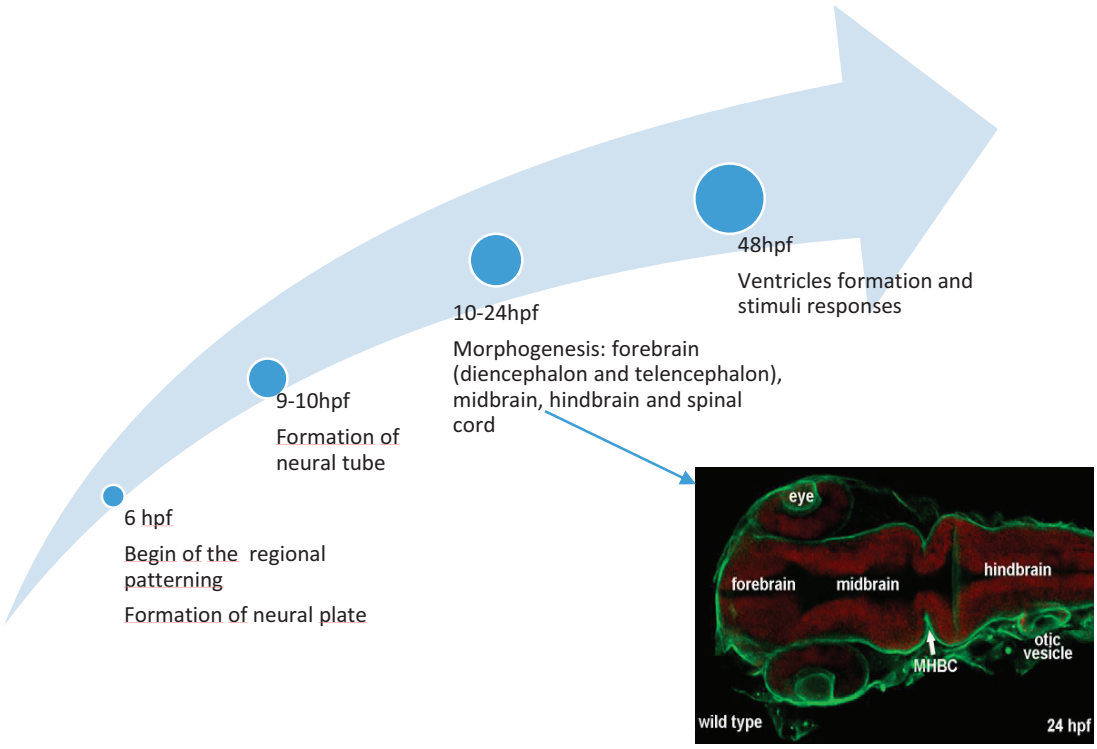


Figure 1: Schematic representation of brain development main events. On the left bottom, a confocal image of 24 hours post fertilization (hpf) wild type zebrafish embryo stained with laminin antibody which outlines the neural tube in green and counterstained with propidium iodide to label the nuclei in red (sivelab.wi.mit.edu/Ongoing%20Projects2.htm, 20/06/2015)

Introduction

As for neurogenesis, the neuronal differentiation starts with the induction of neural progenitors, followed by multiple divisions and pool enlargement, specification and finally differentiation of post-mitotic neurons. (Schmidt *et al.*, 2013) The neural induction, is highly regulated by extrinsic factors (such as Bone Morphogenetic Protein, BMP, Wnt, Fgf) and by intrinsic transcription factors (for example SoxB1 family). Therefore, within the CNS, the distinct cell specification occurs in accordance with the differential expression of molecules, induced by the extrinsic and intrinsic factors. (Papan*et al*., 1999) Neurons connected by axons are found at 48hpf, glial cells from 4dpf. (Brosamle and Halpern, 2002; Kawai *et al.,* 2001) During development, neurogenesis and neurospecification are very important, and the brain is especially vulnerable to insults.

Adult Zebrafish brain still has several proliferation zones (Figure 2), with continuous neuronal renovation, being capable of regeneration and restoration of brain function after sustained injury. (Zupanc*et al.,* 2005; Grandel*et al.,* 2006) Regulatory mechanisms, and proliferation zones are similar between the embryo (after fully formed brain) and adult, but neural derivation is different: embryonic neurons are originated from neuroectodermalephitelium, and adult neurons arise from glial cells. (Schmidt *et al*., 2013)

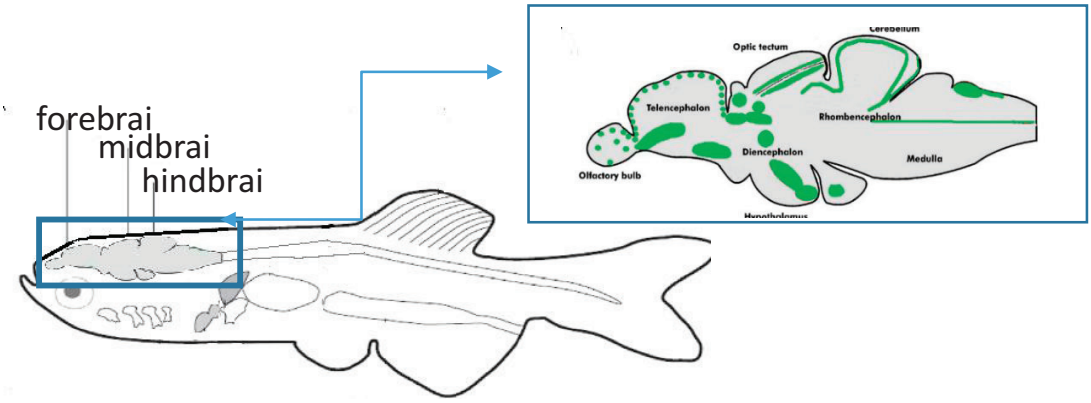


Figure 2: Zebrafish brain: proliferation zones represented in green.

1.4.Anesthesia in Zebrafish

Experimental manipulations in Zebrafish, such as dynamic observations and recording of molecule or organelle movement in cells, are accomplished by the use of anesthesia. (Chen *et al*, 2014) The increased significance of Zebrafish as a model led to more invasive studies and requires longer immobilization periods, resulting in a longer exposition to anesthetics. The monitoring of these studies requires an adequate husbandry and veterinary care to ensure the quality and reproducibility of the results and the well-being of Zebrafish. (Rombough, 2007; Matthews, 2012) However, only few studies were conducted in order to assess the toxicity of anesthetics and its effects in the development of CNS and morphology of Zebrafish.

A good anesthetic "should induce anesthesia rapidly with minimum hyperactivity or stress. It should be easy to administer and should maintain the animal in the chosen state. When the animal is removed from the anesthetic, recovery should be rapid. The anesthetic should be effective at low doses and the toxic dose should greatly exceed the effective dose so that there is a wide margin of safety." (Coyle, *et al*. 2004)

Nowadays, tricaine methanesulfunate (MS222) is the most common anesthetic used in Zebrafish. It eliminates action potentials as well as spontaneous contractions of muscles and simultaneously it can be used to induce sedation, euthanasia, reduction of stress due to transportation, improvement of management, among others. (Ackerman *et al.,* 2005; Alvarez-LaJonchere and Garcia Moreno, 1982) Nevertheless, from the metabolism of MS222 several metabolic residues are released and maintained in circulation long after anesthesia. Recent studies pointed that in experiments that need a long term sedation these metabolites contribute to a high mortality which restricts the use of MS222 especially in studies that require long or repeated anesthesia. (Chen *et al*, 2014; Matthews, 2012) In addition, MS222 also decreases heart rate and may therefore contribute to an increased accidental death risk. (Huang *et al*, 2010)

Recent studies pointed that the effects of MS-222 are dependent on the developmental state of the zebrafish but most of these conclusions were taken by trial-and-error approach and are not conclusive. Nevertheless, Romboughdemonstrated that the use of MS-222 is not safe in larvae due to a reduced liver detoxifying activity and also to the lack of maturated gills (Rombough, 2007). Considering the limitations of MS-222, several studies tried to evaluate the effect of other anesthetics in zebrafish.

One of the anesthetics that was studied was isoflurane, which presented protective effects when administrated with MS-222 but when used alone led to a high and undesirable mortality rate. (Collymore*et al*, 2014)

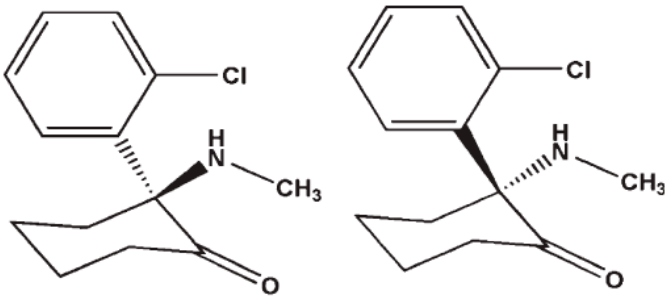
Rapid cooling, which was used for fish euthanasia, was also evaluated as a possible anesthetic. It presented low mortality rates in larval fish and rapid anesthesia however, the mortality was higher in older fish. (Chen *et al*, 2014) In addition, it is not certain that the rapid cooling completely block the nerve conduction, which makes rapid cooling not suitable for invasive procedures. (Ross, 1999; Matthews, 2012)

Clove oil was pointed as a good alternative for the MS-222, due to the ability to induce a quick and safe anesthesia with a lower mortality rate. (Grush*et al*, 2004) However, according to the FDA clove oil contains methyleugenol that is suspected to be carcinogenic and presents detrimental effects in the liver. Therefore, clove oil is not a good alternative to MS-222. (FDA, 2002; Matthews,2012)

In spite of these studies, there is no information regarding the best anesthetic and currently the best option available for Zebrafish anesthesia is still MS-222. Therefore, more detailed studies are needed to improve animal handling quality and to establish a safe and effective anesthesia. A possible alternative to MS-222 is Ketamine, known to have a wide margin of safety. However, the existing studies are controversial regarding ketamine's effect on brain development and consequently a more detailed study of ketamine is needed.

1.5.Ketamine: General Overview

Previous studies considered ketamine as a non-competitive N-methyl-D-aspartate receptor (NMDA-R) antagonist. Ketamine derives from aminocyclohexanone and has a molecular weight of 237Da. It has two optical stereoisomers: (S)-(+) and (R)-(-) that have distinct affinity for the NMDA-R. The S(+) is pointed as being three to four times more potent than R(-) but both enantiomers have a similar pharmacokinetic behavior (similar half life time (180min)). The racemic mixture, which is the most used, is made in acidic solution (pH 3.5–5.5), isfreely water-soluble, and presents a pKa of 7.5. (Kharasch*et al.,* 1992)



R-(–)- ketamine *S*-(+)- ketamine

Figure 3: Ketamine optical stereoisomers: (S)-(+) and (R)-(-)

Ketamine has a half-life of 180 min, in humans. (Clements *et al.,*1982) It is metabolized in the liver predominantly by N-demethylation, by CYP3A4 , CYP2B6, and CYP2C9 into norketamine, and finally dehydronorketamine. (Sinner and Graf, 2008) Norketamine is also biologically active, approximately one-third to one-fifth as potent as ketamine. Dehydronorketamine is less studied, and its effects are not fully understood.

For long time, Ketamine was considered a dissociative anesthetic due to the ability to induce an electrophysiological dissociation between the limbic and cortical systems. Nevertheless, after nearly 50 years of widely medical use, ketamine was withdrawn from human anesthesia because the majority of the patients exhibited unusual symptoms after being submitted to it, such as delusions, hallucinations and confusion. These symptoms led to the investigation of ketamine toxicity and in 1999 a study performed by Ikonomidou et *al*. suggested that ketamine may exert a potential neurodegenerative effect in the developing brain due to neurotoxicity. (Ikonomidou*et al.,* 1999; Pai and Hending, 2007)

However, recently studies pointed that the role of ketamine in the CNS is not completely understood: some studies even defend that ketamine has a neuroprotective effect and may contribute to synaptogenesis (Himmelseher*et al.,* 1996), namely after brain injury. (Wang *et al.,* 2012; Zhou *et al*., 2006) Considering these studies and also the

fact that ketamine is the only anesthetic available that has analgesic, hypnotic and amnesic effects, ketamine is still used to induce anesthesia in several procedure such as cardiac, abdominal, oral and pediatric surgeries; to control pain after major surgery in association with opioids and more recently ketamine started to be used in the treatment of depression. (Morgan, 2012; Duman*et al.*, 2012; Koike *et al*., 2011).

Considering the different studies it appears that according the dose, the brain health state and CNS development, ketamine may act in different signaling pathways and may exert different effects. Therefore, in order to potentiate the use of ketamine a detailed knowledge of its molecular mechanism is needed

1.5.1.Molecular Mechanism of action

For long time, it was considered that ketamine was a non-competitive NMDA-R antagonist since it interacted with the phencyclidine (PCP)-binding site of the NMDA-R complex located within the ion channel, thereby blocking the transmembranous ion flux and blocking the sensory input to higher centers of the CNS exerting its anesthetic and psychotomimetic effects. (Craven, 2007; Morgan, 2012; Bergman, 1999; MacDonald *et al.,*1987)

In parallel studies, it was also put in evidence that ketamine when used in high doses (anesthetic doses) may also have a neurotoxic effect. In studies performed with rodents, the blockade of NMDA-R by high dosage ketamine led to poor brain cell survival and physiological outcome. This neurotoxic effect seems to be associated to the fact that upon blockage of NMDA-R receptor on a GABAergic interneuron, the excitatory action of glutamate is inhibited and the GABA neuron is inactivated and does not release GABA. Considering that the release of GABA and its binding to receptors in the second cortical glutamatergic pyramidal neuron normally inhibits glutamate release: thus, the absence of GABA means that the neuron is disinhibited and the release of glutamate become increased inducing an overexcitation and damage of cells, figure 4. (Nishimura and Sato, 1999; Martin *et al.,* 1990; Hirota*et al.,* 1999; Sleigh *et al.,* 2014).

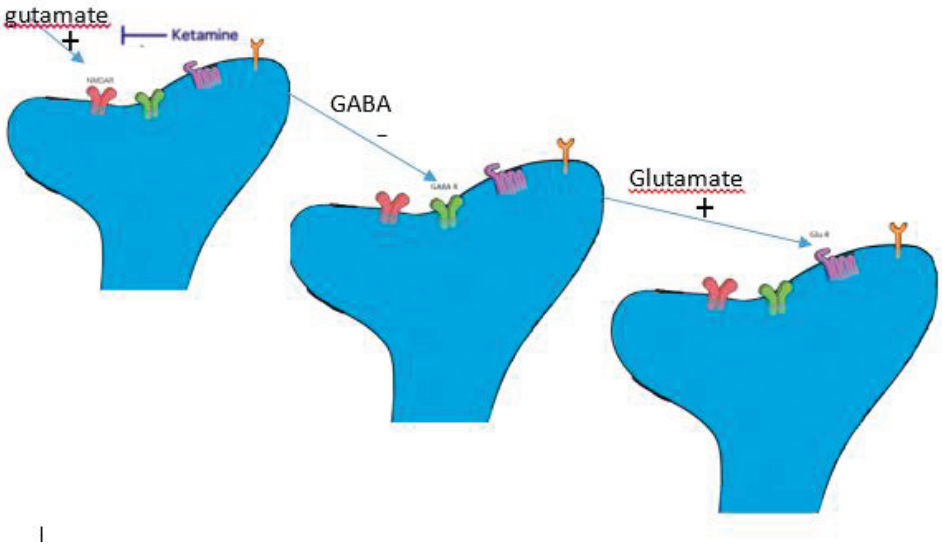


Figure 4: schematic representation of ketamine effect in GABAergic neurons.

However, more recent studies pointed that when ketamine is given at subanesthetic doses, it may relieve depression, suicidal thoughts, and pain in patients who have failed many other treatments. It has been hypothesized that low doses of ketamine could be associated to an increased expression and activity of a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, which stimulate L-type voltage dependent calcium channels (VDCCs) increasing the brain-derived neurotrophic factor (BDNF) release. (Hoeffer and Klann, 2010; Jourdi*et al.,* 2009; Tigaret*et al.,* 2006) BDNF activates AKT and ERK, which stimulates mammalian target of rapamacin (mTOR) pathway, increasing the production of synaptic signaling proteins and the number and function of new spine synapses in the prefrontal cortex, as represented in figure 5 (Livingston *et al.,* 2010; Hoeffer and Klan, 2010; Koike *et al.,* 2011; Mitterauer, 2012) In support of this hypothesis, Li et al. demonstrated that high doses of ketamine did not stimulate mTOR signalling (Li *et al.,*2010).

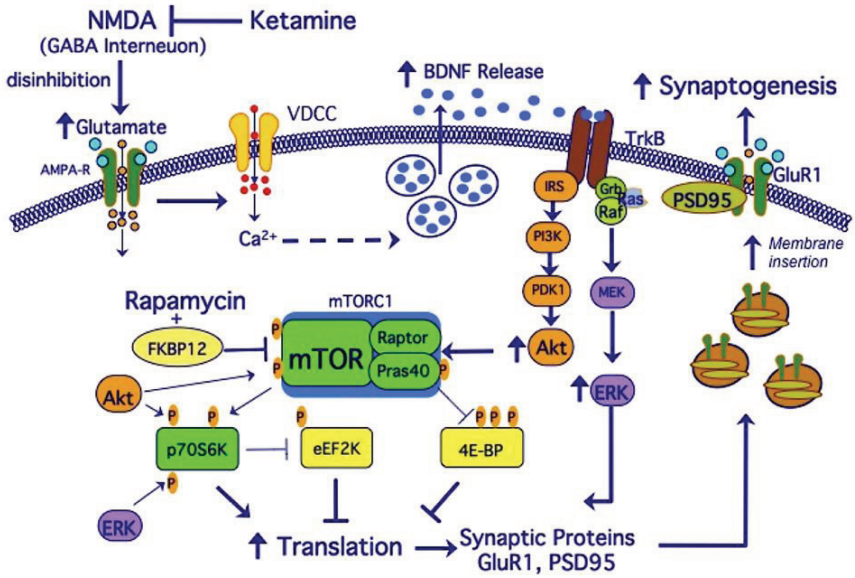


Figure 5: Ketamine mechanism underlying mTOR stimulation, and its antidepressant effect. (Duman*et al.,*2012)

The improvement of synaptogenesis upon the use of low doses of ketamine, may explain why ketamine was effective in attenuating injury resultant from ischemia, and also the improvement in the health state of depressed patients. Together, these results suggested that according the dosage, ketamine could be neurotoxic or neuroprotective. (Diazgranados*et al.,* 2010; Zhan *et al.*, 2001) Nevertheless, considering that high doses of ketamine may present neurotoxic effect, it is important to know the threshold separating beneficial/detrimental effects of ketamine. (Moghaddam, 2002)

In addition, other studies pointed that ketamine may interact with other receptors affecting a wide range of cellular processes: neuronal hyperpolarisation-activated cationic currents (Ih, also known as hyperpolarisation-activated cyclic nucleotide channels (HCN1)), nicotinic acetylcholine ion channels, delta and mu-opioid agonism and opioid potentiation, the nitric-oxide (NO) cyclic guanosine-mono-phosphate (cGMP) system, and metabotropic glutamate receptors (mGluR). (Lydic and Baghdoyan, 2002; Yamakura*et al*., 2000; Kubota et *al*., 1999; Wang et *al.*, 2012) However, the mechanism of action associated to these receptors it is not completely understood. Taken together, these works suggest that upon receptor binding ketamine will produce alterations in several signaling pathways, and its effects are more complex than previously proposed. The interactions of ketamine and possible outcomes are summarized in the table 2.

Table 2: Interactions of ketamine and its possible outcomes. Adapted from Moghaddam et al., 1997 and Duman, 2012)

Channelinteractions	Neuromodulatoryinteraction	Gene expression	Cellulareffects
NMDA-R, AMPA-R, GABA,HCN1, nACh-R, VDCC, opioid channels, Dopamine D_2 receptor, serotonin 5-HT_2 receptors	Modulates Glutamate, Dopamine, Noradrenaline, Acetylcholine, and Opioids levels; AMPAR, NMDAR1 insertion and expression	Interferes with mTOR, BDNF, GFAP expression	Interferes with synaptic maturation, number and stability

1.5.2.Ketamine in the developing brain

Neurotoxicity is the ability of a compound to induce brain damage (transient or sustained loss of function, number). Ketamine neurotoxic effect in the developing brain was first studied in 1999 (Ikonomidou et *al*.,1999) and since then it was assessed in several models: rats, monkeys, rabbits, human and in some cell cultures.

As previously described the ketamine-associated neurotoxicity seems to be associated to an increased release of glutamate. However, the mechanism is not fully understood and previous studies pointed that the neurotoxic effect in the developing brain is slightly different from the mechanism in fully developed brain. Based on current hypothesis it is hypothesized that the blockage of the NMDA–R by high doses of ketamine may alter the normal production and distribution of glutamate which may affect the regular neurogenesis, differentiation and proliferation of neuronal precursor cells, contributing for neurodegeneration. (Benitez-Diaz *et al*., 2003; Miranda-Contreras *etal*., 2000). Furthermore, several studies reported that high doses of ketamine lead to upregulation of one subunit of NMDA-R, the NR-1 which can result in cell death in a calcium dependent manner due to the activation of pro-apoptotic genes, although the mechanism is still unclear(figure 6). (Wang et *al*, 2006; Dong and Anand.,2013; Liu *et al.* 2011).

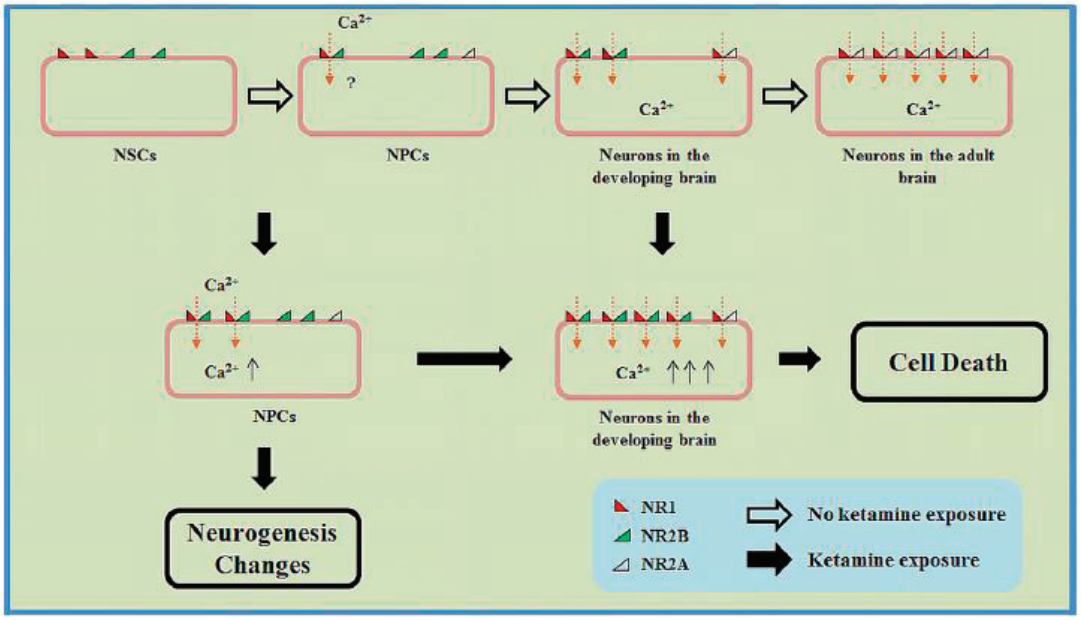


Figure 6: Neurotoxic effects of ketamine on Neuronal Precursor Cells (NPC) and Neuronal stem cells (NSC) in the developing brain. (Dong and Anand,2013)

In the brain cortex, the neuron degeneration and cell death associated to the increased apoptosis, increased expression of Bax, suppression of ERK and Akt phosphorylation has been demonstrated through the treatment of rodents with prolonged and repeated doses of ketamine. (Ullah*et al.,* 2012; Turner *et al*., 2002; Liu *et al*., 2011; Zhang *et al*., 2011; Huang *et al*., 2012; Huang *et al*., 2015) In addition, the studies performed in younger rodents concluded that the younger the animal the more accentuated was the neurotoxic effect, even in lower doses. (Edler, 2012-not published) However, other studies also in rodents, within the same age range showed that there was not an increase in apoptosis compared to control animals. (Lyall*et al*., 2009; Anand*et al.,* 2007; Fredrikssone*t al.,* 2007)

In Rhesus Monkeys ketamine induced apoptosis, more accentuated in fetal monkeys than in neonatal ones. However, this effect was only observed after 3h of exposure to ketamine. (Brambrink*et al.,* 2012)

Taking together, these reports put in evidence that the neurotoxicity associated to the use of ketamine is controversial and seems to be dependent on the dosage, duration of the treatment, and age at the exposure moment. (Soriano *et al.,* 2010; Wang *et al*., 2008; Shang *et al*., 2007; Desfeux*et al*., 2010; Shibuta*et al.,* 2006; Slikker*et al.,* 2007; Dong *et al*., 2013)

1.6.Ketamine- Zebrafish studies

In Zebrafish few studies of the effect of ketamine were published. In 2011, Zachary *et al*. performed a behavior*al* and molecular analysis of ketamine's effect on adult Zebrafish. The results from this study pointed that 0.8% ketamine in aquaculture water was the optimal concentration for anesthesia. In this study it was demonstrated that ketamine exposure affected respiratory and stress response to hypoxia, by a NMDA receptor-dependent mechanism. In addition, the behavior studies upon anesthesia demonstrated that ketamine induced abnormal movements (figure 7). The results were consistent in every application, with no evidence of desensitization or tolerance gain. This was the first evidence that ketamine affects spontaneous behavioral activity. (Zakhary*et al*., 2011)

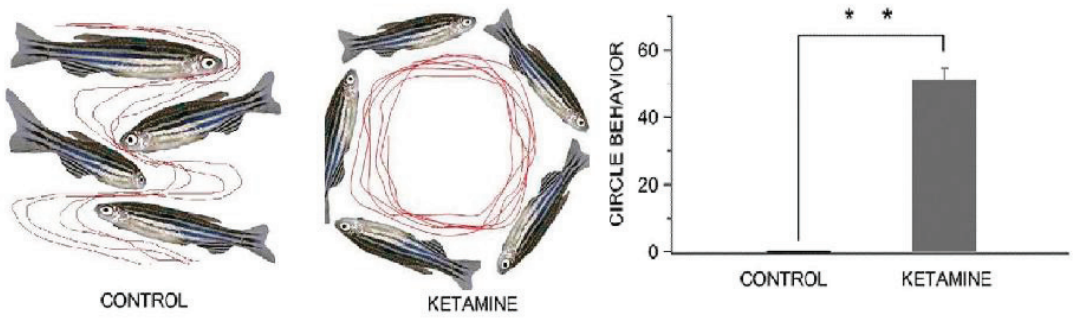


Figure 7: Behavior an*al*yses of Zebrafish exposed to 0.2% of ketamine for 5min. (Zakhary*et al*., 2011)

More recently, the effect of ketamine in zebrafish motor neuron toxicity was evaluated. Transgenic embryos (hb9:GFP-motor neurons identified by GFP expression) , 28hpf, were exposed to 0.5mM and 2mM concentrations of ketamine for 2h or 20h. Several genes involved in neuronal development were selected and analyzed post ketamine exposure and compared to a drug naïve group. The results showed that the exposure to 2mM ketamine for 20 hours resulted in a decreased motor neuron population, but 0.5mM and less exposure had no effect (figure 8).

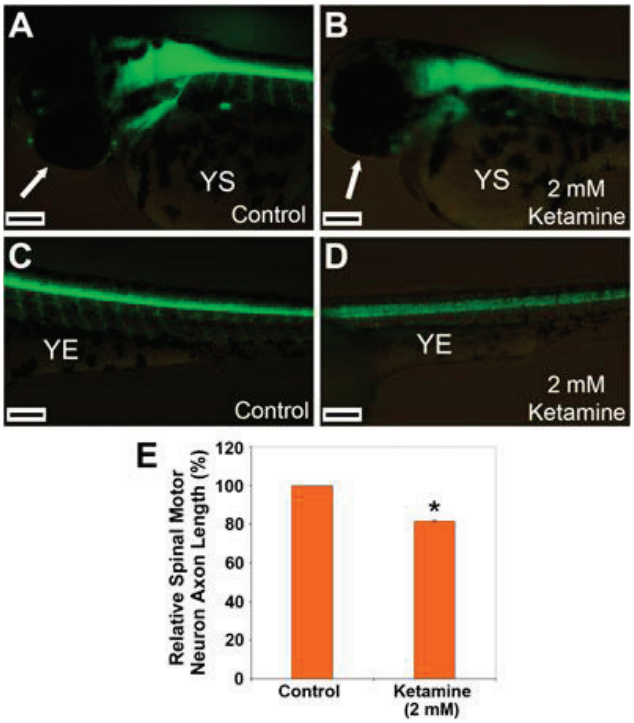


Figure 8: Live imaging of 48hpf embryos with dors*al* side up and anterior side left. Effects of 2mM ketamine for 20h exposure on the over*al*l levels of GFP. YE indicate the yolk extensions and YS yolk sac. The arrows is pointing to the eye. A and B are the brain and C and D the spin*al* cord. Ketamine treated embryos show a reduced over*al*l GFP fluorescence, suggesting a decrease in neuron*al* populations. The differences in length were measured (E) and we can see that ketamine exposure results in a reduced axon length (Kanungo*et al*., 2011)

In addition, this study, showed that ketamine (0.5mM) might favor neuronal commitment but has a negative impact in neuronal differentiation and survival. No direct effects on the morphology were observed. (Kanungo*et al*., 2011)

In conclusion, Kanungosuggested that ketamine exerts neurotoxic effects in the developing brain. These effects were only evident upon the use of ketamine 2mM (approximately 0.05%), which is a lower concentration than that is usually used (0.8%). Nevertheless, this study did not investigate the long-term effect of ketamine in order to determine if the effects were reversible or attenuated.

Regarding the effect of ketamine on the Zebrafish morphology, several studies were performed. (Félix *et al*., 2010; Félix *et al*., 2014). In 2010, Félix and his colleagues used embryos in a prior stage (2-3hpf) and tested 0.2, 0.4 and 0.8% (8mM, 16mM and 32mM respectively) ketamine doses (sub-anesthetic and anesthetic) for 20 min. At 24, 48, 72 and 144 hpf, embryotoxicity and morphologic characteristics were assessed. The results showed that mortality and overall morphological characteristics were similar among groups and the control. But, after hatching, skeletal deformities were found in all

ketamine exposed embryos, suggesting a noxious role of ketamine in morphological development of Zebrafish embryos. (Félix et *al*., 2010)

More recently, the same group published a more complete study of ketamine's effect in Zebrafish embryonic development and its possible toxicity. In this study, it was pointed that the effect of Ketamine in embryos could be mediated by an NMDA independent mechanism, (Félix *et al*., 2014) as exposure to ketamine occurred prior to its appearance on the brain. (Cox, 2005)

In this study, Felix and coleagues used an experimental model similar to that used in the first study regarding the concentrations, exposure times and time checkpoints (figure 9).

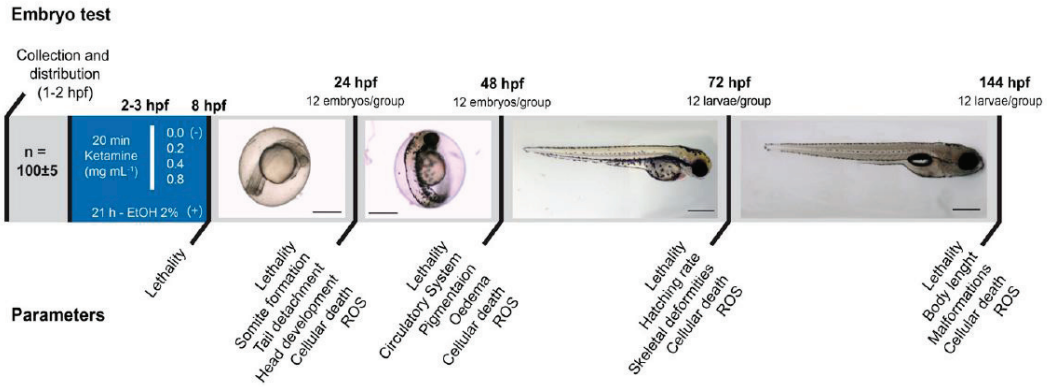


Figure 9: Experimental design used to determine ketamine's effect in Zebrafish embryonic development (Félix *et al*., 2014)

The mortality observed in this study was ketamine dose dependent, reaching a peak when embryos were exposed to 0.8mg/ml. With this ketamine dose it was reported an increase in the mortality rate between 8 and 48hpf, after that the mortality was constant, but always higher than that observed in the control group (figure 10).

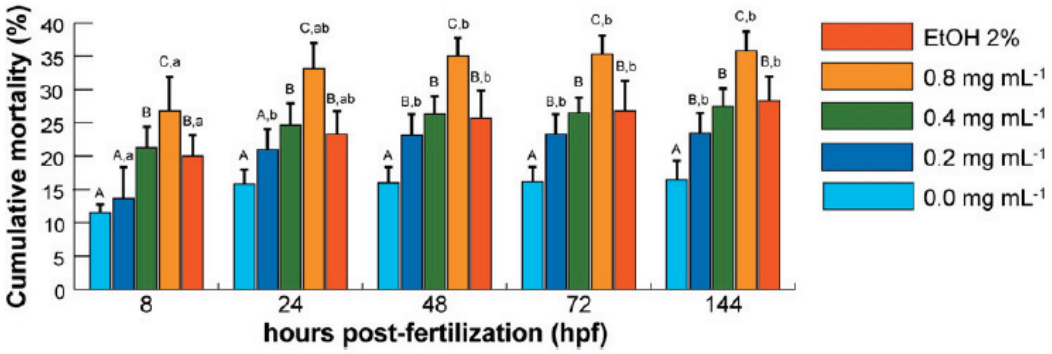


Figure 10: Mortality rate of Zebrafish exposed to ketamine and ethanol (positive control). Mortality rate was higher in ketamine treated groups in a dose dependent manner.

Regarding the toxic effects in the development, the study showed that in 24 hpf, ketaminetreated embryos (all doses) the only parameter affected was the tail detachment from the yolk sac, which was lower than that observed in control embryos. However, in the 48 hpf, ketamine (0.8 mg/ml) treated embryos it was observed edema of the yolk sac and of the heart. Nevertheless, at 72hpf, ketamineexposure did not affect significantly hatching but a tendency was noted: decreased hatching with increased ketamine concentration.

When 3hpfzebrafish embryos were exposed to 0.8 mg/mL of ketaminesevere malformation on Zebrafish were detected in larvae (144hpf), such as enlarged organs, namely the heart, undershot jaw (figure 11). In addition, there was also abnormal distribution of melanocytes on affected groups (positive control and highest dose of ketamine).

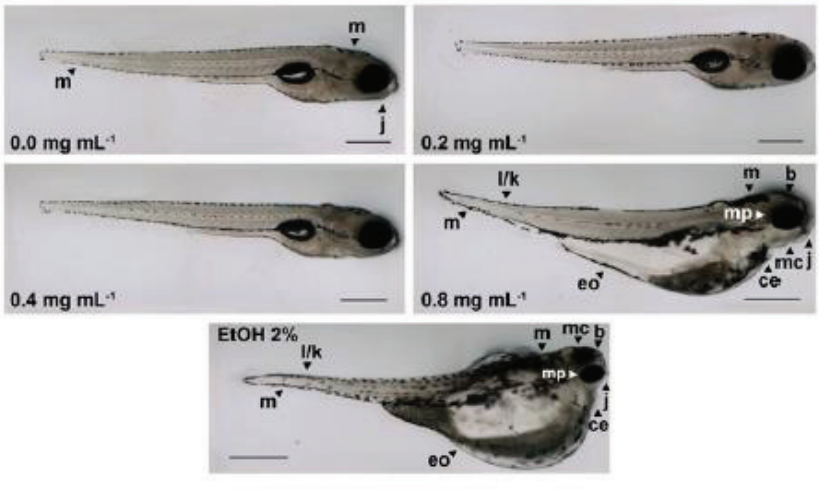


Figure 11: M*al*formations of Zebrafish exposed to ketamine and ethanol. Arrowheads point the major spots where the most noticeable m*al*formations were. Ce-cardiac enlargement, b-brachyceph*al*y, j-undershot jaw, mc- microceph*al*y, mp-microphth*al*mia, eo-enlarged organs, l-lordosis or k-kyphosis,m-melanocytes.(Félix *et al*., 2014)

Previous studies reported the occurrence of apoptosis after ketamine exposure. (Scallet*et al*., 2004; Brambrink, 2012) However, in the study performed by Félix et al. it was not reported the presence of apoptotic cells. Considering that there were no significant differences from the other studies and also that the main difference is the age at which Zebrafish were exposed to ketamine it was hypothesized that at this stage (before 7hpf) Zebrafish embryos do not express receptors involved in the occurrence of apoptosis (Félix *et al*., 2014)

In conclusion, this study showed that early ketamine exposure (blastula phase) is toxic to the embryos and affects their normal development, increasing the incidence of malformations and also mortality. These effects seem to be NMDA-R independent, since at this stage the NMDA-R expression is not described. (Félix *et al*., 2014) Nevertheless, this study did not considered the effect of ketamine at long term, and therefore it did not evaluate if the exposure in the blastula phase could be associated with other alterations in adult fish.

Considering all the results obtained with ketamine it seems that this anesthetic it is not the most adequate. However, all the anesthetics available have negative effects and it should be weighed which in fact is worse, since none is perfect. Anesthesia interferes with regular signaling and it is somewhat expected to have some noxious effects. It is important to understand the possible extrapolation to humans (no mortality is described and morphologic effects are not visible) and also how to better understand the exact effects and extent of ketamine exposure to validate the technique and reduce variability in result interpretation (in studies that require anesthesia this is crucial).

More studies are needed to assess proliferative and apoptotic effects of ketamine and also to better understand the mechanism by which ketamine acts and the effective role of NMDA receptors in its mechanism of action. A better understanding of these mechanisms underlying ketamine action might be helpful to develop a protective technique and decrease the teratologic effects of ketamine, taking the most advantage of its analgesic and anesthetic features.

2.Objectives

Given the necessity of finding an adequate anesthetic to use on zebrafish research, the present work aim to evaluate the effect of ketamine on CNS cell proliferation and apoptosis.

The available literature, regarding the effect of ketamine on CNS, is controversial. The studies were not performed in the same stage of Zebrafish development, the concentration of ketamine vary from one study to another, as well as the duration of ketamine exposure. Therefore, it is quite difficult to attain both the mechanism of action of ketamine at the molecular level, and its effects.

The present work aimed to:

- Assess the effect of ketamine in different developmental stages. This was studied by exposure of zebrafish at different stages of development to ketamine: gastrula, blastula and segmentation; and to different concentrations (0,2mg/ml; 0,4mg/ml; 0,8mg/ml for 20 min and 0,05mg/ml; 0,07mg/ml; 0,09mg/ml for 24h)

- Evaluate the effects of ketamine over time, after exposition. Thus, after the exposition, zebrafish were divided in different groups in order to evaluate the effect of ketamine over time and collection were made at 34hpf, 50hpf and 144hpf.

- Assess the effects of ketamine. Here, embryos and zebrafish larvae from each group were used to determine the proliferation potential (by PCNA expression and BrdU incorporation), to evaluate the occurrence of apoptosis (by caspase-3, AIF expression and TUNEL), autophagy (by LC3 expression), and pluripotency (by SOX 2 expression). Pathways thought to be involved in ketamine response were studied by the expression of effector proteins: phospho-AKT, phosphor-ERK, PKC. Expression studies were made by western blot and immunohistochemistry.

3.Material and methods

3.1.Chemicals

Ketamine (Imalgene 1000, 100 mg/mL) wasobtainedfromMerial Portuguesa-Saúde Animal Lda (Rio Mouro, Portugal). Solutions were prepared daily with system water at 28.5ºC; 200 mg/mL instant ocean salt water and 100 mg/mL sodium bicarbonate, UV sterilized. In all experiments the plastic and glass material as well as solutions were sterilized by autoclaving at 121 ° C or by filtration using a 0.22 µm pore size mesh. DNA isolation kit (high pure PCR template preparation kit), biotin-16-dUTP and terminal transferase, protease and phosphatase inhibitors were purchased from Roche. Phalloidin was supplied from Life science technologies (Thermoscientific). PVDF membrane was purchased from Milipore. Dithiothreitol and methanol were acquired from VWR, Prolabo, Belgium. ECF, western blotting Reagent Pack, was acquired from GE Lifesciences, Pittsburg, PA. Acrylamide (MB04501), was purchased from Nzytech. Immunohistochemistry reagents (Ultra V Block solution, biotinylated secondary antibody and enzyme labelled streptavidin) were purchased from Labvision (Thermoscientific). Antibodies were purchased from Thermofisher, Cell Signalling, Sigma-Aldrich and Abcam as described in Table 3.Mowiol was purchased from Calbiochem. All other chemicals were obtained from Sigma- Aldrich (Steinheim, Germany).

3.2.Maintenance of Zebrafish and embryo collection

All experiments were performed according to European and Portuguese legislations on animal welfare. Wild type Zebrafish (AB strain) were kept at a water temperature of 28±0.5 ◦C and under a photoperiod of 14:10h (light:dark), in a semi-closed aquaria with a recirculation system with both mechanical and biological filters. Adequate water hardness (3-4 dGH) was ensured by the addition of sodium bicarbonate. Supplementar air was provided to keep the oxygen levels close to saturation values. The fish were fed twice a day with a commercial fish diet (Sera, Heisenberg, Germany) supplemented with *Artemia*sp. *nauplii.*

Embryos were obtained from spawning adults grouped overnight, in tanks. The beginning of light period in the morning induced spawning and newly fertilized eggs were collected and rinsed several times in a system water before random distribution by experiment. System water was substituted daily.

3.3.Exposure of zebrafish to ketamine

Considering that the main objective of this project was to determine if the exposure to ketamine may alter the development of the CNS, zebrafish were exposed to different concentrations of the anesthetic at different stages of development and were then

collected and analyzed at different periods of time (figure 12)The ketamine concentrations and time of exposure were chosen accordingly previous studies where ketamine effects on Zebrafish were already reported (Reimers et al., 2004; Riehl et al., 2011; Zakhary et al., 2011). Therefore, exposure to ketamine occurred at 2hpf (blastula stage), 5.5hpf (gastrula stage) and 10hpf.

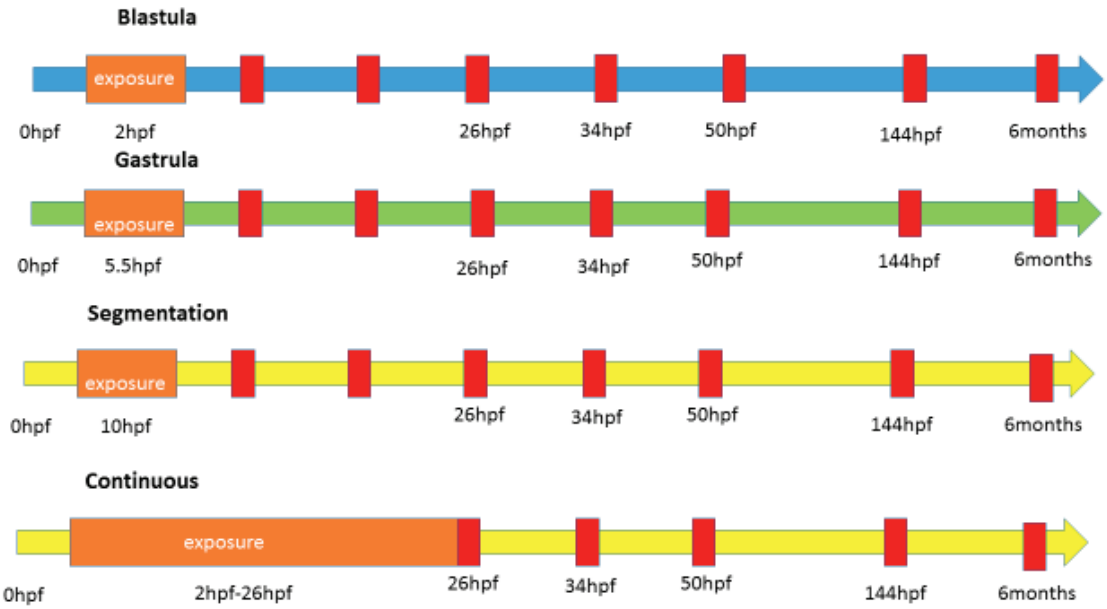


Figure 12: Design of the project, indicating the moment at which zebrafish were exposed to ketamine and the time at which zebrafish were then collected for analysis. The red marks represent collection points.

Zebrafish embryos in blastula, gastrula and segmentation were subdivided according the ketamine concentration, in four different groups:

- Negative control: no chemical exposure
- Animals exposed to 0.4 mg/mL ketamine
- Animals exposed to 0.8 mg/mL ketamine
- Animals exposed to 0.2 mg/mL ketamine

Additionally, there was a group of zebrafish that was continuously exposed to ketamine between 2-26hpf. In this group, animals were also subdivided in four groups according the ketamine concentration:

- Negative control: no chemical exposure
- Animals exposed to 0.05 mg/mL ketamine

- Animals exposed to 0.07 mg/mL ketamine
- Animals exposed to 0.09 mg/mL ketamine

Before the exposition to ketamine embryos were analyzed and those with apparent normal development were selected. Approximately 100 embryos per group (blastula, gastrula and segmentation) were placed for 20 min, in 50ml beakers containing different ketamine concentrations (0.2, 0.4 and 0.8 mg/ml). At the end of the exposure period embryos were washed three times in system water and allowed to develop until the time point for the collection as mentioned in the figure 12.

3.4.Evaluation of the Zebrafish morphology

After zebrafish collection, embryos and larvae were fixed in ethanol 70% for 24h, dehydrated and embedded in paraffin. Thin tissue sections (3 μm) were cut, mounted on glass slides, immersed in xylene (twice for 10 min) followed by rehydration with graded ethanol (5 min in 100%, 5 min in 95%) and transferred to deionized water. In order to evaluate the integrity of the tissue sections and to study the zebrafish morphology, three sections from each animal were stained with hematoxilin / eosin according a previous protocol. (Fehr *et al*., 2015)

3.5.Evaluation of cellular proliferation, apoptosis and autophagy by immunohistochemistry

Immunohistochemistry was used to identify cell populations and also to evaluate cell proliferation, apoptosis and autophagy. For that the antibodiesindicated in Table 3 were used.

Zebrafish paraffin sections were rehydrated as previously described. Endogenous peroxidases were blocked with H_2O_2 for 30min, and then washed twice in Tris-buffered saline with Tween 20 (TBS-T contained 20mM Tris, 137 mMNaCl, 0.1% Tween 20, pH 7.6). To achieve antigen retrieval tissue sections were immersed in citrate buffer (0.1M sodium citrate, 0.1M citric acid, pH 6), heated in the microwave (2 times, 5 minutes, 400W) and then washed with TBS-T. To block non-specific background staining, slides were incubated with Ultra V Block, 10min at room temperature, then probed with appropriate primary antibody overnight at 4ºC (see table 3). Negative and positive controls were performed simultaneously. In the negative control, the primary antibody was replaced by PBS. For the positive control it was used tissue sections previously evaluated for the expression of the specific protein.

Secondary biotinylated antibody (Goat anti-polyvalent), followed by enzyme labeled streptavidin (horseradish peroxidase) and chromogenic substrate (DAB) were used. Slides were then washed, counterstained with hematoxilin, dehydrated with xylene and mounted with DPX.

Computer-assisted inverted biomicroscopy (Zeiss) was used to capture the immunostaining images using the same light intensity and color thresholding for all sections of each stain.

3.6.Evaluation of cellular proliferation, apoptosis, autophagy by Western Blot

In order to quantify the expression of proteins associated to proliferation, apoptosis, autophagy and the amount of the phosphorylated PKC, ERK1/2 and Akt, zebrafish embryos were stored in PBS at -80º and thaw immediately before each experiment. PBS was discarded, lysis buffer was added (RIPA buffer: 150nM NaCl, 1% (v/v) Triton X-100, 0.5% (W/v) sodium deoxycholate, 0.1% (w/v) SDS, 50mM trizma base, 2mM EDTA, pH 8.0) and supplemented with protease and phosphatase inhibitor cocktails and DTT 1mM , followed by a brief sonication (3 pulses of 2'', 180 watts). Homogenates were incubated 30 min on ice, protein concentration was determined using the bicinchoninic acid and then the proteins were denatured. For that, denaturation buffer (0.25M trizma base (pH 6.8), 200mM DTT, 20% (w/v) glycerol, 4% (w/v) SDS and 0.05% (w/v) bromophenol blue) was added to each cell lysates at a 1:1 ratio and then exposed to 95°C, for 5 min. Thereafter, 30 µg of total protein was added to each lane of a 12% SDS-PAGE, and separated by electrophoresis (buffer solution contained by 50mM trizma base plus 50mM bicine and 0.1%(w/v) SDS) and transferred (transfer buffer solution contained 12.5mM trizma base, 96mM glycine, 20% (v/v) methanol and 0.1% (v/v) Tween) to a previously activated PVDF membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T contained 20mM Tris, 137 mMNaCl, 0.1% Tween 20, pH 7.6), probed with appropriate primary antibodies overnight and alkaline phosphatase(AP)-conjugated secondary antibodies. All antibodies used in this work are listed in Table 3.The imunocomplexes were visualized by chemifluorescence using enhanced ECF and a typhoon FLA 9000 system (GE Healthcare Bioscience AB, Uppsala, Swesen). Densitometric quantification was performed in unsaturated images using Total Lab (Nonlinear dynamics, Lta.). Immunoblotting, using anti-actin were used to confirm comparable loading of the interest protein in each lane. Each experiment was repeated three times.

Table 3: List of primary and secondary antibodies used for the western blot and immunohistochemistry

Antibody	Host/Clonality	Application	Dilution	Company
anti-PCNA	Mouse Monoclonal	WB/IHC	1:100	Vector Laboratories
anti-LC3	Rabbit polyclonal	WB/IHC	1:250/1:800	Thermofisher
anti-caspase 3	Rabbit	WB/IHC	1:250/1:300	Cell signaling
anti-sox2	Rabbit	WB/IHC	1:500/1:200	Abcam
anti-AIF	Rabbit	WB/IHC	1:250/1:200	Cell signaling
anti-PKC	Rabbit	WB	1:1000	Cell signaling
anti-pERK	Rabbit	WB	1:1000	Cell signaling
anti-pAKT	Rabbit	WB	1:1000	Cell signaling
anti-p38	Rabbit	WB	1:1000	Cell signaling
anti- GFAP	Mouse	IHC	1:100	BD Biosciences
anti- actin	Mouse	WB	1:2500	Millipore
anti-tubulin	Mouse	WB	1:2500	Sigma-Aldrich
anti-laminin	Mouse	WB	1:1000	Sigma-Aldrich
anti-calnexin	Goat polyclonal	WB	1:2500	SICGEN
anti-GAPDH	Goat polyclonal	WB	1:2500	SICGEN

3.7.Evaluation of cellular proliferation using bromodeoxyuridine (BrdU)-colorimetric assay

In order to determine the cellular proliferation at CNS of zebrafish upon treatment with ketamine, we adapted in our laboratory a method to quantify the BrdU incorporation. For that, embryos were exposed to ketamine as previously described, 24h after ketamine administration they were exposed to 10 mMBrdU for 20min, according previous studies. Six hours after that embryos were collected in PBS. A second group of embryos were exposed to BrdU only at 138hpf and collected in PBS at 144hpf. Embryos and larvae were frozen at -80º and thaw immediately before each experiment. In order to determine the incorporated BrdU, the DNA content of embryos and larvae was determine

using a DNA isolation kit and then the incorporated BrdU was quantified according a cell proliferation ELISA kit.

-DNA isolation (high pure PCR template preparation kit)

Zebrafish embryos and larvae were lysed by sonication in lysis buffer and then incubated at 55ºC with proteinase K for 1h. DNA isolation was performed according to kit instructions. After DNA isolation, a DNA precipitation solution was added (2 Volumes of cold 100% Ethanol and 1/10V of 3M sodium acetate), incubated overnight at -80º, centrifuged in order to precipitate and the supernatant discharge.

-BrdU detection

The isolated DNA was resuspended and incubated in a solution with the ability to fix and to denature the DNA (Cellular Proliferation ELISA kit) for 1h, at room temperature. Afterwards samples were centrifuged 30min at 20.000rpm and supernatant discarded. Anti-BrdU-POD solution (Cell proliferation ELISA kit) was added (1:100) and incubated for 2h at room temperature. Samples were then centrifuge, washed with PBS, incubated with substrate solution (Cell proliferation ELISA kit), for 25min and absorbance was read at 370nm (492nm reference). Controls without DNA, and with zebrafish that were not exposed to BrdU were made to validate the results.

3.8.Evaluation of cellular proliferation using BrdU by confocal microscopy

With the purpose of evaluating the distribution of BrdU incorporation, embryos were exposed to ketamine and BrdU as described above. After collection, they were fixed in PFA 4% for 4h and then transferred to PBS. Upon usage embryos were rinsed in distillated water and permeabilized in acetone (-20º), for 25minutes, and further washed in 0.09M Phosphate Buffer. To decrease background, incubation in Sodium Borohydride (1µg/ml solution in PBS) was made for 5 minutes. Afterwards, samples were incubated for 1h in 5% BSA (w/v solution in PBS). Incubation with anti-Brdu primary antibody (1:1000 in 1% BSA solution in PBS, cell signaling) was conducted overnight. Secondary antibody, Alexa488 (1:200) in 1%BSA solution was added for 2h, room temperature. Embryos were then mounted in mowioland images were collected by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany).

3.9.Evaluation of apoptosis

In addition to the use of anti-caspase antibody, apoptosis was evaluated using Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay which is based in the capability of TdT to bind to blunt ends of degraded DNA. It is generally used to detect apoptotic cells that suffer extensive DNA degradation. (Kyrylkova*et al*., 2012) For that, tissue sections previously rehydrated, were washed with PBS and incubated 30min with 0.25% Triton in PBS. Afterwards, TdT buffer (30 mMTris-Cacodylate, Terminal tranferase, dUTP, Cobalt(II) chloride) was added and incubation proceeded for 1h, at 37ºC. After washing with TB (Tris-Borate) buffer (30mM sodium citrate, 300mM NaCl), slides were incubated with fluorescein (1:100) for 1h, and then with DAPI (5ug/ml). For controls,

TdTbuffer was omitted. The images were collected by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany).

3.10.Evaluation of F-actin organization

Considering that ketamine may interfere with cell cytoskeleton,the organization of F-actin was evaluated. For that, it was used Phalloidin that is a bicyclic peptide belonging to a family of toxins isolated from the Amanita phalloides mushroom with high affinity to F-actin. To evaluate F-actin organization, tissue sections from zebrafish embryos and larvae were labeled with Alexa-Fluor 555-Phalloidin. For that, rehydrated tissue sections were washed with PBS, permeabilized with Triton 0,1% in PBS, for 20min. After washing with PBS, the incubation with Phalloidin (1:500 in PBS) and DAPI (5ug/ml) proceeded for 30min. Slides were then rinsed and mounted using an anti-fade mounting medium. To determine the autofluorescence of the tissue sections Alexa-Fluor 555-Phalloidin was omitted. The images were collected by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany).

3.11.Statistical analysis

Data presented in this work is representative of at least 3 independent experiments. Data was analyzed by standard computer programs (GraphPad Prism 6 for Windows, version 6.01, GraphPad Software, Inc.) and is expressed as mean ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnet's test. Differences were considered significant at $P<0.05$.

4.Results

The results presented in this section were obtained according to the methods previously described for Zebrafish. However, there were some limitations probably associated to the samples that limited the acquisition of results. All the procedures proposed by the project were completed, but only a few had results that could be reliable. The limitations and problems are further explained in the troubleshooting section.

4.1.Organization of Zebrafish Central Nervous system

To study the histology of the CNS, 3µm tissue sections obtained from control zebrafish and from zebrafish treated with ketamine were stained with hematoxilin-eosin and observed under a light microscope. The analysis of the tissue sections revealed that several zebrafish embryos and larvae presented alterations in tail and curvature, and in others it was possible to detect edemas. However, due to the occurrence of several artifacts associated to the tissue processing, it was not possible to associate these deformities to the treatment with ketamine, since they were observed in ketamine treated and non-treated zebrafish embryos and larvae.

In spite of the artifacts, it was possible to identify in CNS of 144hpf zebrafish larvae (ketamine treated and non-treated) five major areas: telencephalon, diencephalon, mesencephalon, rhombencephalon and spinal cord (figure 13). In each area, various subareas were identified: olfactory bulb in telencephalon, hypothalamus and pretectum in the diencephalon, tectum opticum in the mesencephalon, cerebellum and medulla oblongata in the rhombencephalon (data not shown). (Grandel*et al*., 2006; Wullimann*et al.*, 2009)

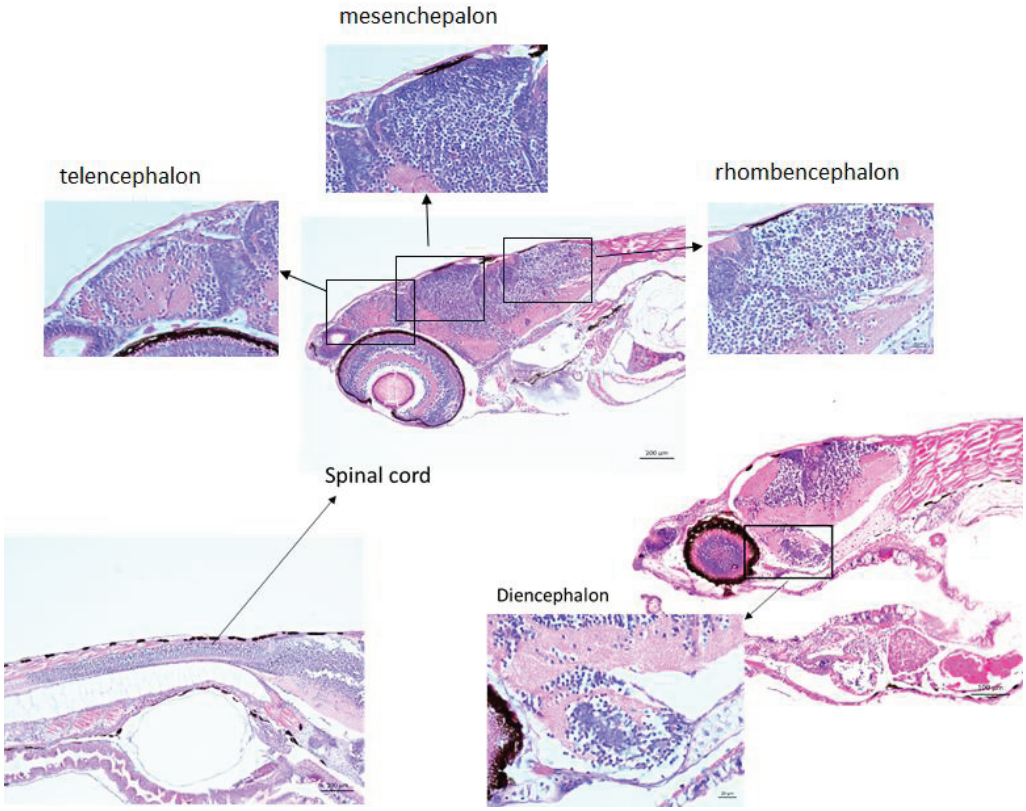


Figure 13: Main areas of Zebrafish brain (144hpf larvae), from control group. Tissue sections were stained with hematoxilin-eosin. Magnification 100x, scale bar 100µm; 400x scale bar 20µm

4.2.Effects of ketamine in cell proliferation ability

Previous studies have reported the effects of ketamine in cell proliferation and its contribution to the development of CNS. However, these results constitute a matter of intense debate. *In vitro* studies pointed that, upon 6h of ketamine exposition, there was an increased in the proliferation potentialof neural stem cells. (Bai *et al.*, 2013) Nevertheless, a study performed *in vivo* referred that ketamine may suppress neural stem cells proliferation. (Huang *et al.,* 2015). However, the dosage of ketamine and the methods used to evaluate proliferation and survival were different in both studies, and therefore the comparison is not straightforward. Considering that ketamine is a frequently used anesthetic, it is important to clarify its role on CNS cell proliferation, and its overall impact on CNS development. To address that, different doses of ketamine were administered at different stages of zebrafish development, in order to achieve a complete characterization of ketamine's effect in the CNS development. Cell proliferation was

measured by two different methods: BrdU incorporation and Proliferation Cell Nuclear Antigen (PCNA) detection.

4.2.1.Evaluation of the incorporation of BrdU

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is an analog of thymidine, and it is incorporated into DNA during the S-phase of the cell cycle. BrdU incorporation was assessed using an ELISA-like colorimetric assay, after DNA isolation and denaturation as previously described. To evaluate the influence of ketamine in different stages of development, zebrafish embryos were organized into four different groups according to table 4.

Table 4: Experimental design of the BrdU experiment

Stage of development	Time of exposure	Ketamine dosage (mg/ml)	BrdU exposure (10mM)	Zebrafish collection points
Blastula (2hpf)	20min	0,0/0,2/0,4/0,8	26hpf	32 and 144hpf
Gastrula (5.5 hpf)	20min	0,0/0,2/0,4/0,8	29.5hpf	35.5 and 144hpf
Segmentation (10hpf)	20min	0,0/0,2/0,4/0,8	40hpf	46 and 144hpf
Continuous (2-26hpf)	24hpf	0,0/0,05/0,07/0,09	40hpf	46 and 144 hpf

In embryos exposed during the blastula stage (2hpf) and collected at 32 and 144hpf, there were no statistical differences regarding the incorporation of BrdU for the different ketamine dosages, in both time points. However, in zebrafish collected at 32hpf, the incorporation of BrdU was higher than that detected at 144hpf (figure 14).

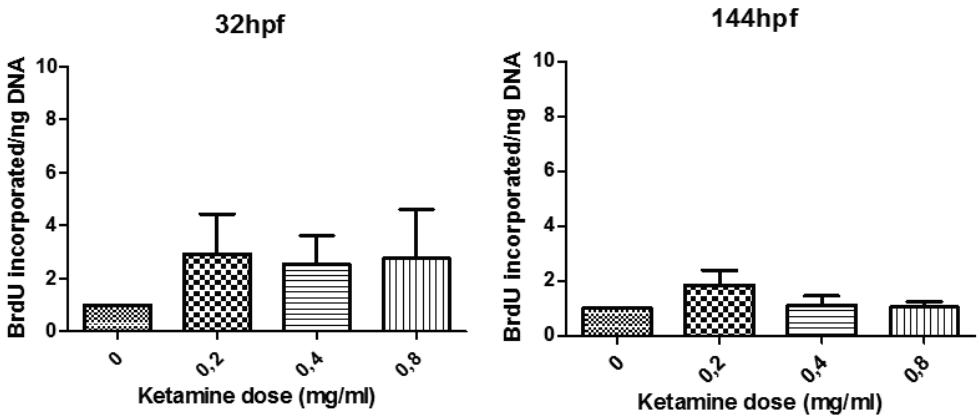


Figure 14: Evaluation of BrdU incorporation per ng DNA, in embryos exposed to ketamine (20 min), during Blastula stage (2hpf) and collected at 32 and 144hpf. All values were mean ± SEM, each one performed in triplicate.

In zebrafish embryos exposed to ketamine at 5.5hpf (Gastrula stage) and collected at 35.5hpf and 144hpf, there were no statistically significant differences regarding the incorporation of BrdU for the different ketamine dosages and collection points. The incorporation of BrdU in ketamine treated zebrafish embryos was similar to that detected in control embryos (figure 15).

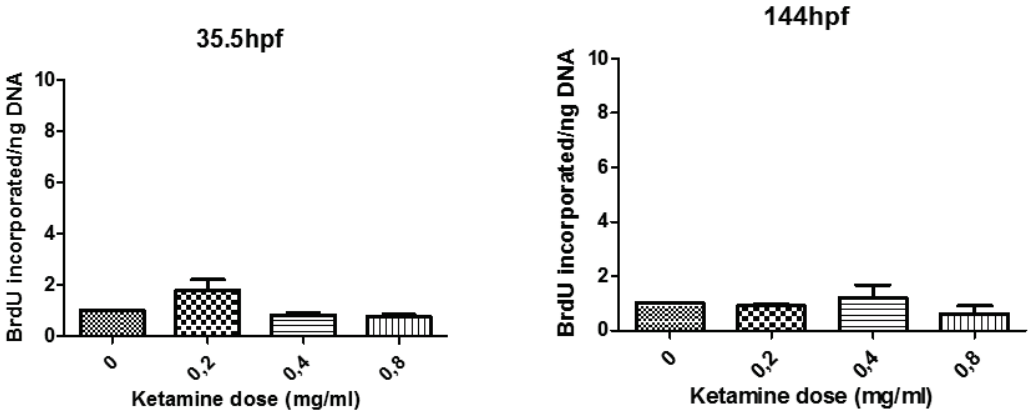


Figure 15: Evaluation of BrdU incorporation per ng DNA, in zebrafish embryos exposed to ketamine (20 min) at 5.5hpf (Gastrula stage) and collected 35.5hpf and 144h. All values were mean ± SEM, each one performed in triplicate.

Regarding zebrafish embryos exposed to ketamine during segmentation (10hpf) and collected at 46hpf and 144hpf, the results also showed that there were no statistical

differences in BrdU incorporation. However, in larvae collected at 144hpf, the incorporation of BrdU was higher than that detected at 46hpf (figure 16).

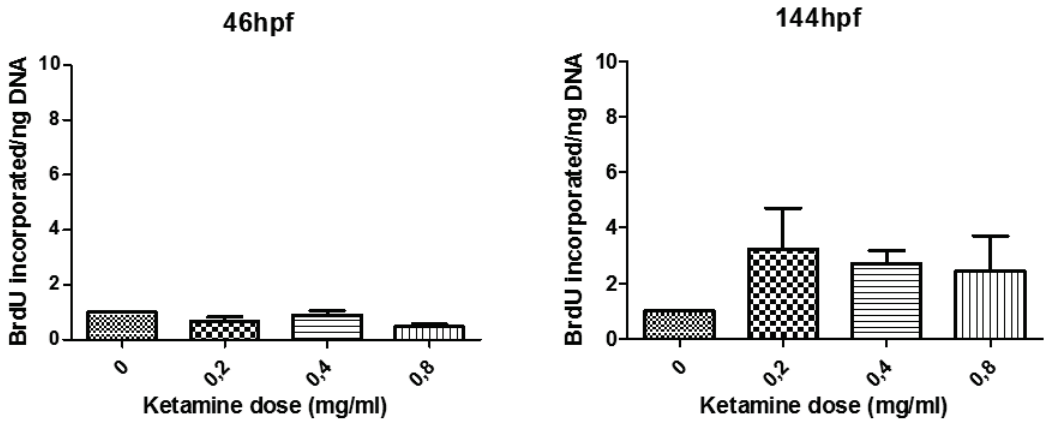


Figure 16: Evaluation of BrdU incorporation per ng DNA, in zebrafish embryos exposed to ketamine (20 min) during segmentation (10hpf) and collected at 46hpf and 144hpf. All values were mean ± SEM, each one performed in triplicate.

In the zebrafish group exposed for 24h to ketamine, (from blastula stage, 2hpf until 26hpf) the results showed that the incorporation of BrdU was higher than that detected in control embryos, but also not statistically significant (figure 17).

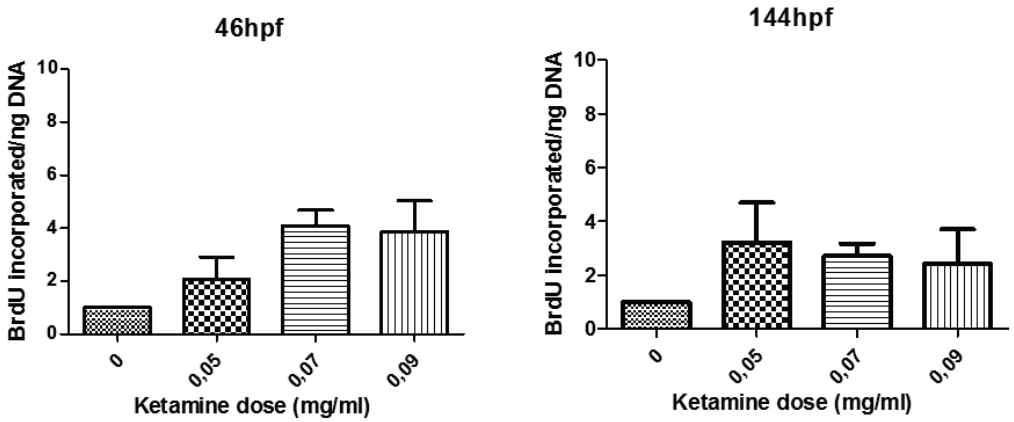


Figure 17: Evaluation of BrdU incorporation per ng DNA, in zebrafish embryos exposed to ketamine for 24h and collected at 46hpf and 144hpf. All values were mean ± SEM, each one performed in triplicate.

4.2.2.Evaluation of PCNA expression

PCNA is a DNA clamp involved in DNA replication and repair mechanisms. (Kelman, 1997; Moldovan *et al*., 2007) The protein encoded by this gene is found at the nucleus and is a cofactor of DNA polymerase delta that increases the processivity of the leading strand synthesis during DNA replication. It is known that, in response to DNA damage, this protein is ubiquitinated and is involved in the RAD6-dependent DNA repair. (Moldovan *et al*.,2007)

Considering that the expression of PCNA is correlated with cell proliferation, in the present study, PCNA expression was only evaluated in zebrafish embryos exposed to ketamine during segmentation stage (10hpf), collected at 34, 50, and 144hpf. The evaluation of the levels of PCNA expression was performed by western blot and immunohistochemistry. The western blot was used to quantify the PCNA expression levels, whereas immunohistochemistry was used to evaluate the pattern of its expression (PCNA distribution along the tissue)

The PCNA expression was evaluated by western blot in embryos collected at 34hpf and 50hpf, and in larvae collected at 144hpf as shown in figure 18. No statistical significant differences were found regarding the time points of collection, nor ketamine concentration, which is consistent with the results previously obtained with BrdU.

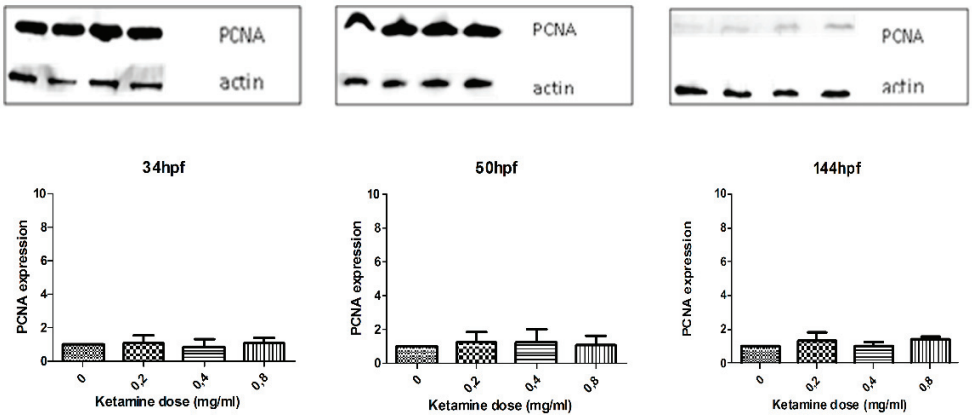


Figure 18: Detection of PCNA expression by Western Blot. Zebrafish embryos exposed to different concentrations of ketamine during segmentation stage (10hpf), and collected at 34, 50, and 144hpf. Samples were probed with antibodies against PCNA and actin was used as loading control. All values were mean ± SEM,each one performed in triplicate.

Results

In spite of the fact that no changes in proliferation were detected, regarding the incorporation of BrdU and the PCNA expression, it was hypothesized that the pattern of the CNS proliferation areas could be altered during ketamine exposure. Therefore, the expression of PCNA was evaluated through immunohistochemistry. For that, zebrafish embryos were exposed to ketamine during segmentation, as previously described and then the larvae were collected at 144hpf. However, differences regarding the tissue sections quality made the detection of the immune complexes difficult to analyze and no differences between ketamine treated and non-treated zebrafish were detected. Nevertheless, the expression of PCNA was detected in areas that were previously described as proliferative areas, namely dorsal telencephalic proliferation zone, tectal proliferation zone and mesencephalic lamina in the mesencephalon, and cerebellum (figure 19).

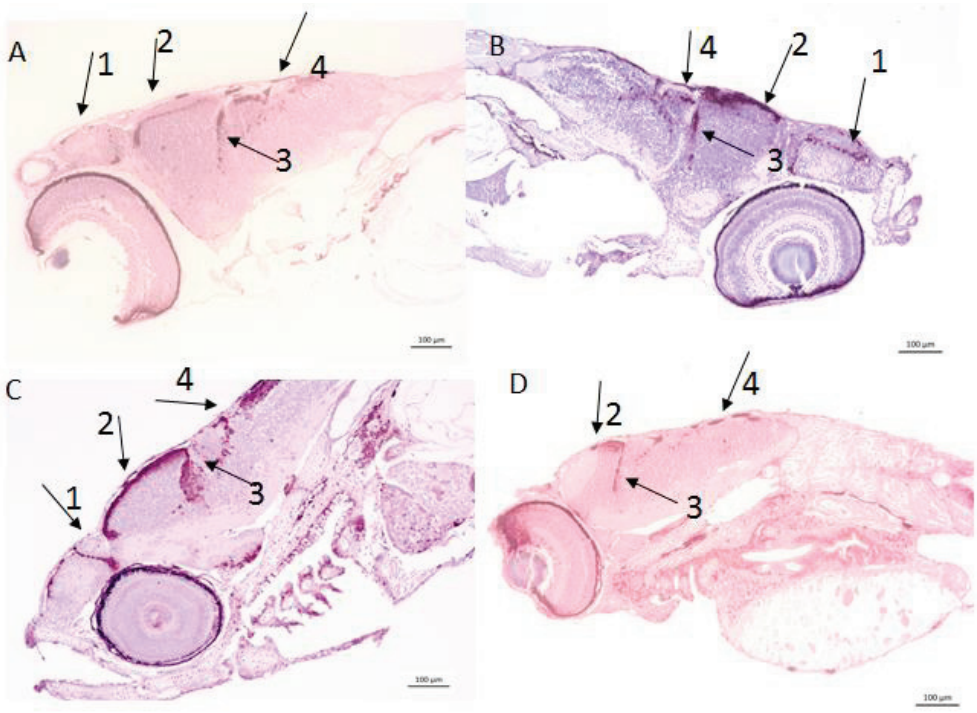


Figure 19: Evaluation of PCNA expression by immunohistochemistry in Zebrafish exposed to ketamine in segmentation stage (10hpf) and collected at 144hpf. **(A)**no ketamine, **(B)** 0.2mg/ml ketamine**, (C)** 0.4mg/ml ketamine and **(D)** 0.8mg/ml Ketamine. **1**-dorsal telencephalic proliferation zone, **2**-tectal proliferation zone, **3**-mesencephalic lamina, **4**-cerebellum. . Magnification 100x, scale bar 100µm.

Altogether, the results obtained through the analysis of BrdU and the expression of PCNA suggest that ketamine does not affect cellular proliferation in the CNS. Considering that the cell proliferation and renewal is dependent on the existence of neural stem cells, we evaluated the expression of Sox 2, a transcription factor expressed in various types of embryonic and adult stem cells, by western blot and immunohistochemistry. However, due to tissue artefacts in immunohistochemistry, and to the absence of a loading control in western blot it was not possible to present those results.

Taking into consideration that previous studies pointed that anaesthetic doses of ketamine may induce apoptosis, DNA degradation was evaluated by TUNEL assay, the expression of caspase-3 and the expression of the apoptosis inducing factor (AIF) were evaluated by western and immunohistochemistry. (Ullah*et al*., 2012; Turner *et al*.,2012) As described, to perform the TUNEL assay tissue sections of zebrafish larvae were incubated with TdT buffer in order to detect DNA degradation. In addition the caspase-dependent apoptosis was evaluated through caspase-3 that is considered an executioner caspase that, upon activation, suffers a conformational change that brings the two active sites of the caspase dimer together, creating a functional mature protease that participates in apoptotic events. Moreover, to evaluate the occurrence of caspase-independent apoptosis it was evaluated the expression of AIF.

Despite having used three different techniques to study apoptosis, it was not possible to get a definitive result due to the occurrence of tissues artifacts and also to difficulties to visualize protein bands during SDS-PAGE electrophoresis.

Considering that: 1) ketamine did not altered the proliferative potential of zebrafish cells; 2) it was not obtained a conclusive result regarding the occurrence of apoptosis in embryos; and that 3) ketamine may induce cell damage, it was hypothesized that ketamine may induce cells to activate autophagy as a survival strategy. In order to evaluate the occurrence of autophagy, it was evaluated the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3) which is a soluble protein present in two isoforms (I and II) with a molecular mass of approximately 17 and 19 kDa, respectively. LC3 is distributed ubiquitously in cells however, during autophagy, LC3 is recruited to autophagosomal membranes. (Tanida *et al*.,2008) The expression of LC3 I/II and its ratio, was evaluated by immunohistochemistry and by western blot in ketamine treated and non-treated zebrafish, but no consistent results were obtained.

Regarding the potential protective role of ketamine, some studies report that ketamine positively influences synaptogenesis, via mTOR pathway. Previous studies reported that ketamine increased the expression of phospho-Akt and phospho-ERK 1/2 , the upstream activators of mTOR. (Li *et al*.,2010) Therefore, levels of phospho-Akt and phospho ERK 1/2 in response to different ketamine doses, were studied by western blot. In addition, it was also evaluated, the expression of protein kinase C (PKC), which is involved in cell proliferation and survival. (Yang *et al*.,2011). To evaluate the influence of ketamine in PKC phosphorylation, the phosphorylated levels of PKC were evaluated by western blot. However, due to technical problems it was not possible to achieve reliable results.

In addition to the evaluation of signaling pathways involved in cell proliferation and survival, it was also evaluated the expression of Glial Fibrillary Acidic Protein (GFAP). This protein is an intermediate filament protein which is involved in cell communication, namely astrocyte-neuron communication. Upon noxious stimuli astrocytes can be activated and the expression of GFAP may increase. To verify if ketamine administration leads to astrocyte activation, GFAP pattern of expression was studied by immunohistochemistry, however due to technical limitations it was not possible to present the results.

5.Troubleshooting associated to the use of Zebrafish embryos and larvae

As mentioned previously, in this work several drawbacks occurred and limited the success of the initial project. There were several difficulties associated to the organization of the project that may explain some of the drawbacks: there were not a reliable test to evaluate the zebrafish health state before and upon treatment with ketamine; zebrafish were maintained and exposed to ketamine at the University of Trás-os-Montes and then brought to CNBC in the fixative solution or frozen. In addition to these two difficulties it is also important to consider the difficulties associated to the use of embryos. The following section aimed to explain some of the difficulties, in order to prevent similar difficulties in a future work.

5.1.Troubleshooting associated to the use of embryos

Using zebrafish embryos and larvae was more challenging than initially thought, which brought several problems during the execution of the proposed experimental procedures.

Considering the reduced dimensions of zebrafish embryos and larvae, and also that zebrafish were collected and processed in geographically distant laboratories, one of the main concerns was the fixative. Adequate fixation of biological material is the most important step in histological procedures. A good fixative should have the ability to maintain cellular structure and tissue morphology, while preserving the immunoreactivity, and having anti-germicide properties. At the same time, it is important to have a good balance between structure preservation and antigen availability. Paraformaldehyde (PFA) and alcohol-based solutions are the most commonly used fixatives.

In routine histological procedures, PFA is the most used. It conserves the morphology and cellular details, as required, and avoid the cellular lysis. In addition, short term fixation in PFA is associated to a reduced autofluorescence what makes PFA convenient in studies of fluorescence microscopy. However, prolonged storage in PFA can lead to an increased autofluorescence, and to a decreased ability to detect antigens due to cross-linkage of proteins and consequent epitope blockage. Antigen retrieval reduces the cross-linkage, however, after extended fixation times, it is less efficient and may destroy extracellular epitopes. When storage time is elevated, there are alternatives to PFA, namely alcohol-based fixatives. Ethanol fixation allows the maintenance of the histologic structure, with a reduced autofluorescence, although morphological details are not as well conserved as with PFA. (Su et al., 2004; Webster et al.,2009)

Since during the course of this project, zebrafish were collected at the University of Trás-os-Montes and then brought into our laboratory, zebrafish were maintained in fixative for long periods of time (more than a week) with the subsequent high-risk of overfixation and of increased protein-linkage. For this reason, it was decided to maintain embryos and larvae in PFA for 24h, and then to transfer them into 70% ethanol. Nevertheless, in some samples the fixation was not the most adequate since some areas

were degraded, presenting autolysis (figure 20). The tissue degradation was detected in H&E-stained sections, and also in the whole mount preparations (figure 21).

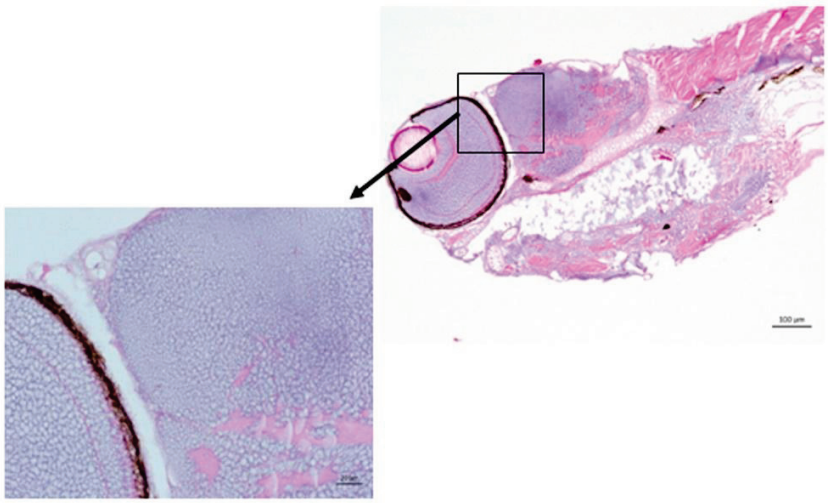


Figure 20: Autolysis is evident in some fish. A deficient fixative process may explain the alteration in tissue hystology. H&E of 144hpf zebrafish. Fish exposed during segmentation to 0,8mg/ml ketamine. Figure is representative, autolysis was verified in all groups. Magnification 100x, scale bar 100µm; 400x scale bar 20µm.

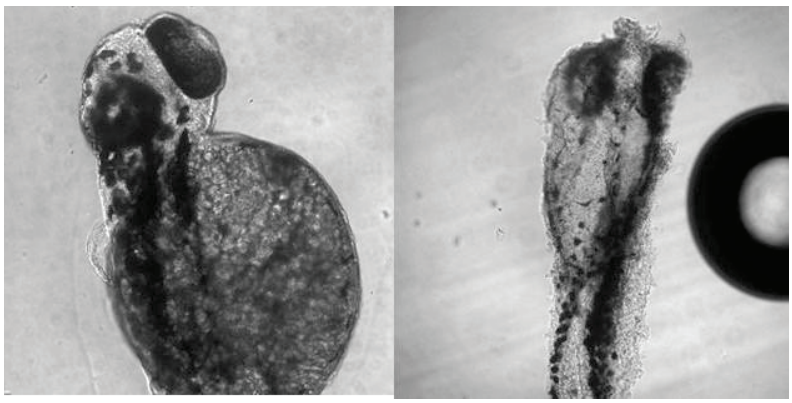


Figure21: Comparison between two zebrafish embryos (46hpf) from control group: on the left, it is shown a zebrafish that was not in a good condition, the shape was altered and structures and tissues areas were not evident. Magnification 100x.

Another difficulty associated with the use of zebrafish embryos was the standardization of tissues sections. The reduced length of zebrafish embryos made them

difficult to process for histochemistry and immunohistochemistry studies (Table 5). In order to avoid mechanical alterations during the fixation and dehydration processing, embryos were carefully manipulated and even then morphological alterations were induced. Furthermore, due to their reduced length, it was difficult to place them with the same orientation in the paraffin block and in the glass slide, which difficult the comparison between tissue sections from different zebrafish. For these reasons, tissue sections from embryos in blastula and gastrula were not used in histochemistry and immunohistochemistry studies and it was decided to use only tissue sections from zebrafish larvae.

Table5: Average length of zebrafish according the development stage

Hour post fertilization	Length
34	2.5mm
50	3.1mm
144	4.2mm

Upon the decision to use tissue section from zebrafish larvae they were embedded in paraffin and sliced into 3um sections. Each section was numbered (first cut, section number one, and so on) in order to allow the comparison between tissue sections from different zebrafish larvae. However, when tissue sections were analyzed, it became evident that zebrafish presented inter-individual characteristics regarding body size and curvature (figure 22 and 23). Considering that ketamine may induce morphological alterations and also that the fixation and paraffin embedding process may also induce some alterations, it was not possible to know accurately the cause of the alterations. These doubts avoid the achievement of one of the primary objectives of this work: to determine whether ketamine induce morphological alterations in the zebrafish CNS.

Furthermore, in order to achieve a standard position and orientation of the tissue sections in the glass slides, technique-related damages, such as twisted fish and striation can be induced, invalidating the use of the given section (figure 24). Consequently, it was difficult to compare the histology of specific areas of the zebrafish larvae and to take conclusions regarding the effect of ketamine since these alterations were visualized in controls and also in ketamine-treated embryos.

In addition, some control embryos and ketamine-treated zebrafish embryos presented edema and alterations in the shape and length of the tail without being possible to known the exact cause (figure 25 and figure 26).

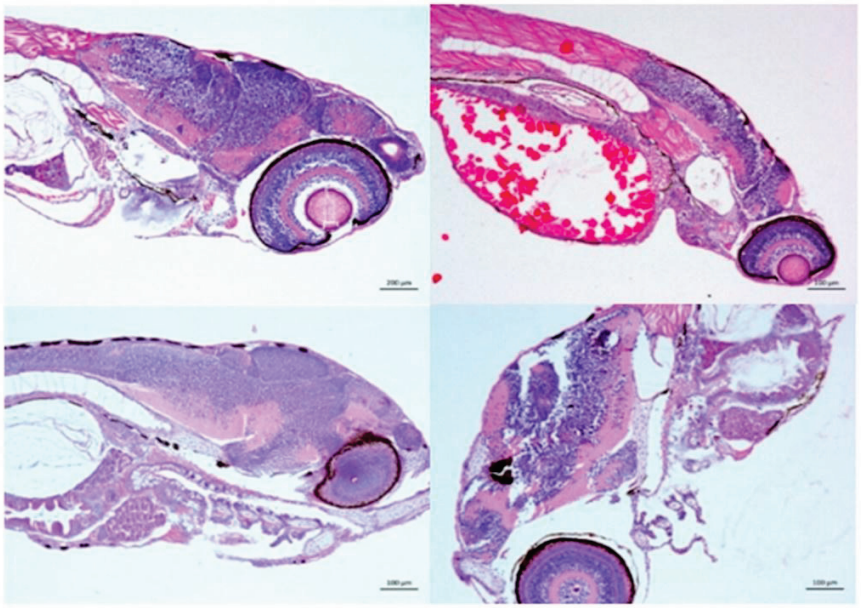


Figure 22: Tissue section from three different control Zebrafish larvae. Same section number (9) in each slide. However, the histological characteristics of the CNS are different. Tissue sections were stained with haematoxilin / eosin. . Magnification 100x, scale bar 100µm.

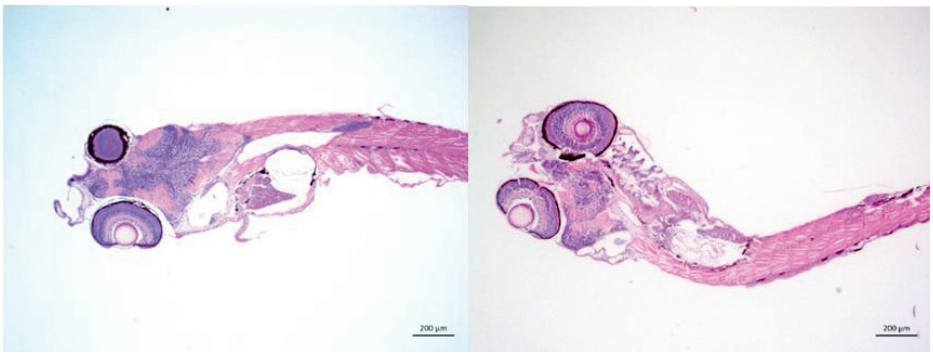


Figure23: Tissue section from zebrafish larvae. Tissue sections were dorsally oriented. The figures obtained from section 9 in both slides represent different layers of the zebrafish and therefore it was difficult to compare its histology. Tissue sections were stained with haematoxilin / eosin.Fish exposed to 0,4mg/ml of ketamine at 10hpf (segmentation). Magnification 50x, scale bar 200µm.

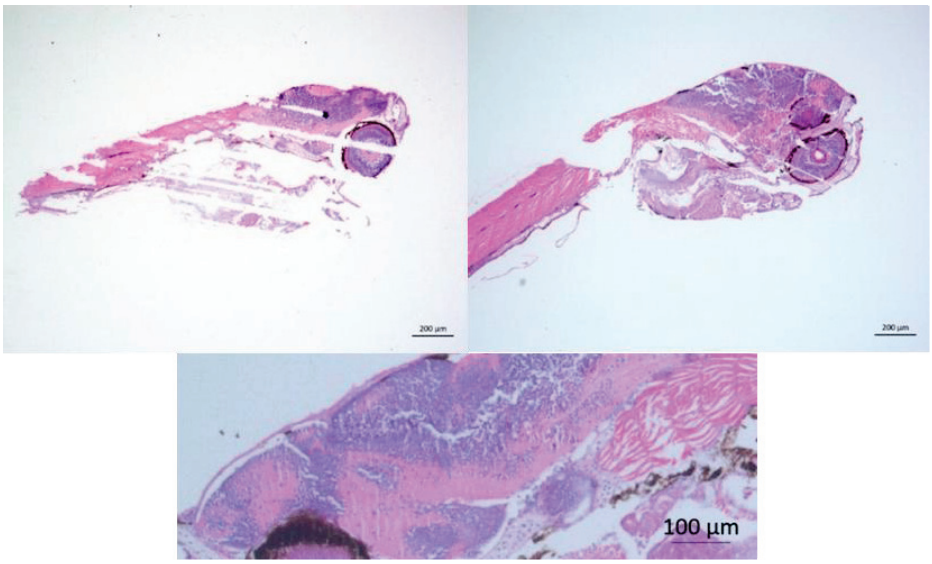


Figure 24: Artifacts resulting from technical manipulation of the tissue sections of 144hpfzebrafish: striation, twisted fish, and sections that lack entire cellular areas can be observed. Zebrafish exposed to 0,2mg/ml (top) and control (bottom). Sections were stained with haematoxilin / eosin. Magnification 50x, scale bar 200µm; 100x scale bar 100µm

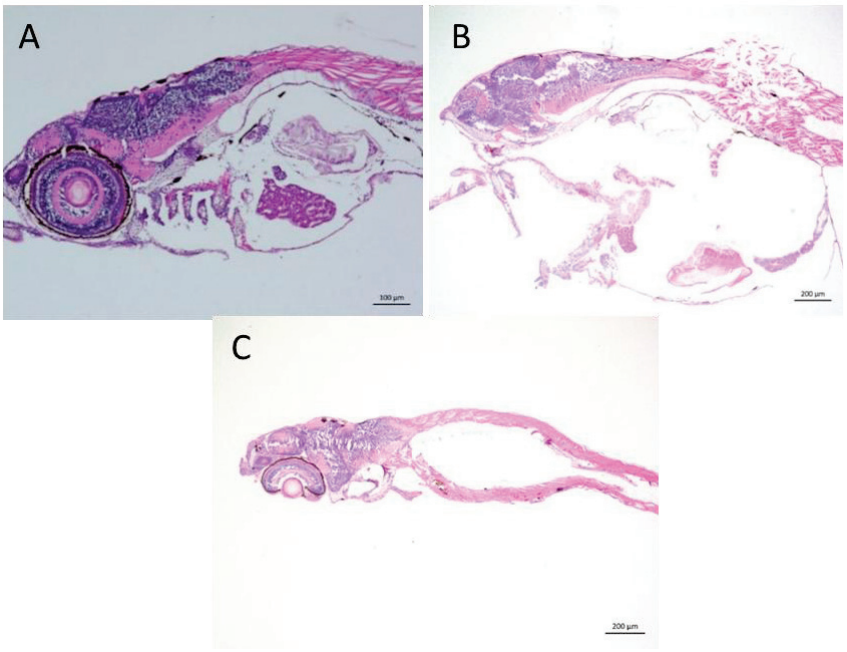


Figure 25: Tissue section from control zebrafish larvae (144hpf) showing some deformities and the presence of alterations suggesting edema. Zebrafish control (A) and exposed to 0,2 (B) and 0,4 mg/ml (C).These deformities were present on every group and were not dependent of ketamine exposure. Tissue sections were stained with hematoxilin / eosin. . Magnification 50x scale bar 200µm; 100x, scale bar 100µm;

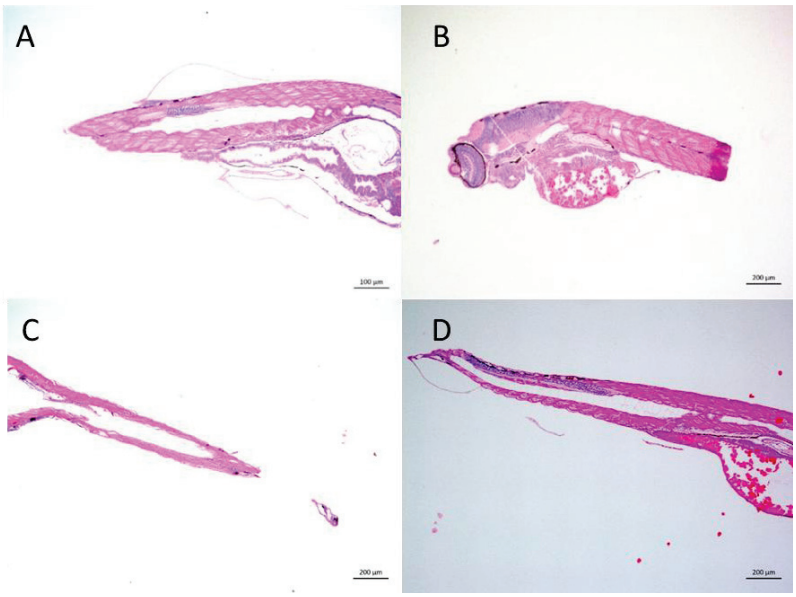


Figure 26: Tissues sections of control (A and D) and 0,4mg/ml ketamine exposed (B and C), zebrafish larvae (144hpf) evidencing differences in the tail. This differences were common to all groups.Tissue sections were stained with haematoxilin / eosin . Magnification 50x, scale bar 200µm.

5.2.Difficulties associated to extraembryonic structures

Zebrafish embryos, developed outside the uterus in association with two structures: the chorion and the yolk cell.

The chorion envelops the developing embryo and it is present until hatching, up to 72hpf. It is a membrane with pores of approximately 0.5 to 0.7 µm diameter, and spaced at 1.5 to 2.5 µm interval. (Lee *et al.,* 2007) The pore canal has a viscosity 26 times superior to the egg water. The chemicals diffusion to the embryo are consequently delayed and therefore function as barrier that may pose difficulties to the diffusion of ketamine. (Ali *et al*., 2011)

The yolk cell is a transient extra-embryonic tissue that arises during early cleavage stages. During gastrulation, the yolk has a critical role in cell fate specification and morphogenesis and also in the nutrition of the embryo. It decreases throughout the development and it is almost empty by 72hpf. (Virta and Cooper, 2011; Kimmel *et al.,*1995) The presence of yolk sac in early stages has been associated with two main issues: one is the increased autofluorescence, that limits the use of fluorescently-

conjugated antibodies in immunofluorescence; and the second one is the total amount of protein that may turns difficult the identification of the embryos proteins in western blot.

To avoid contamination with proteins of the yolk sac, previous studies have suggested that the yolk sac should be removed prior to the tissue processing. (Link *et al.,*2006) The removal is based on the use of pronase (2mg/ml, for 5-10min). However, due to the ratio embryo: yolk size (figure 27), it is difficult to guarantee the entire removal of the yolk, without inducing degradation of embryos, by pronase and other proteases. As shown in figure 25, some of the embryos maintained the yolk sac.

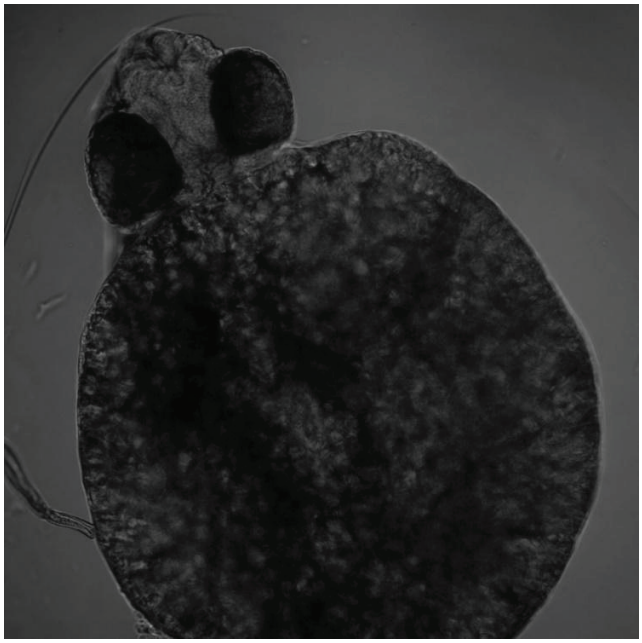


Figure 27: Image representing a yolk sac in a zebrafish embryo (32hpf). The image was obtained in the transmission mode using a control fish. Magnification 200x.

Embryos present a reduced amount of total protein, this oblige to the use of a great number of zebrafish in order to perform the detection of specific proteins by western blot. Bearing in mind that some zebrafish maintained the yolk sac and also that to prepare samples for further western blot analysis the whole sample is sonicated, the proteins from the yolk sac may induce a “dilution” effect in the protein quantification, leading to overrated values. In addition there was a great asymmetry between the protein amount of the samples: some samples presented a great concentration and others presented a reduced yolk sac protein concentration. The variability of protein concentration from different sources (embryo or yolk sac) among each sample lead to significantly different concentrations of the target protein that made difficult its use in electrophoresis. The

results were not consistent between experiments, reveling different amount of proteins within replicas (figure 28).

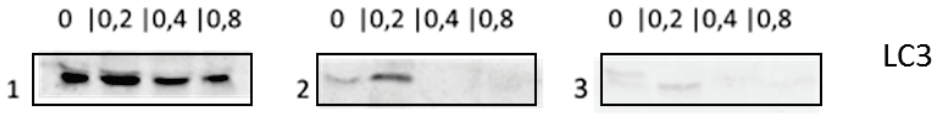


Figure 28: LC3 labeling in 3 independent experiments with the same amount of net protein in each well (30µg), from 12hpf embryos. Each blot was made with different samples.

The yolk sac presented another problem: in the whole mount confocal studies, the yolk has higher autofluorecence when compared to that on ketamine-treated embryo without yolk sac. The removal of the yolk sac (mechanically or with pronase) eliminates the autofluorescence but the procedure associated to the removal, damage the sample and turn difficult the acquisition of good images.

5.3.Troubleshooting associated to immunohistochemistry technique

Immunohistochemistry was performed to evaluate in the zebrafish CNS, the expression and distribution of several proteins, such as LC3, AIF, PCNA, sox-2, caspase-3, and GFAP. However, quantification and comparison of the number of positive cells between groups was difficult. Different sections have different brain areas that may present a different pattern of staining. This variation could be observed in sections stained for PCNA. PCNA is expressed in specific regions. When looking at different sections, the presence/absence of those regions will result in different labeling, which is independent of the treatments performed.

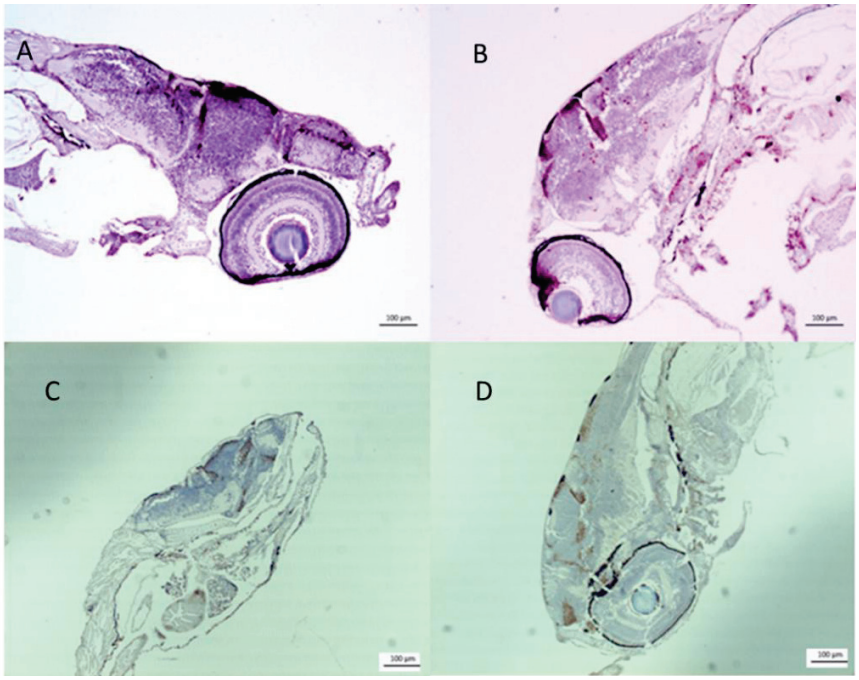


Figure 29: Immunohistochemistry for PCNA detection Zebrafish non-treated (A and D) with ketamine, Zebrafish treated with 0.2mg/ml ketamine (B), and zebrafish treated with 0.8mg/ml ketamine (C). Different brain areas are presented in each section, which makes a comparison difficult. . Magnification 100x, scale bar 100µm.

5.4.Troubleshooting associated to Western bloti technique

Western blotting is a relatively simple and straightforward technique but it depends on an amount of factors such as protein concentration, denaturation buffers, acrylamide concentration and antibody dilution. Thus, its optimization to proteins from different species is sometimes a long and difficult process. In this project, the first difficulty was to gather enough animals to obtain detectable levels of protein. The highest amount of protein that it was possible to use was 40µg protein/lane, considering the volume limit. However, this limit results in low signal for several proteins. This problem could have been easily solved by increasing the number of zebrafish in each sample. However, given the amount of animals we had available at that time, that it was not possible. Another difficulty was the contamination of embryos protein by the yolk protein, as already presented.

Considering that western blot is a semi-quantitative technique, we tried to quantify the expression of several proteins in cell lysates obtained from zebrafish exposed to ketamine and collected as previously described (table 3): LC3, caspase-3, AIF, Sox 2, phosphor-Akt, phosphor-ERK 1/2, and phospho-PKC. Despite the use of antibodies specific for zebrafish antigens, the detection of imunocomplexes was difficult in most of the times. In order to ensure that proteins were transferred from the gel to the membranes, gels

were stained with Coomasie Blue, while membranes were stained with Ponceau. In parallel, positive controls were performed, and the antibodies concentration were increased without a significant improvement in the results.

To test sample quality, we tried to detect proteins from cytoskeleton expressed by all cells: actin, tubulin, calnexin, GAPDH, lamin. Previous studies pointed that ketamine did not alter the expression of these proteins. However, in most of the samples it was not possible to detect immunocomplexes for any of the antibodies nor in control samples nor in samples from ketamine treated zebrafish.

To assure that protein concentration was not limiting the results, it was made an effort to obtain more zebrafish per sample and several protein concentrations were tested: 30, 40, 60 and 70µg of total protein loaded were evaluated. The best results were obtained with 30µg (better defined bands, no unspecific labeling and less background). Proteins that were not detected with 30µg were not detected with 70µg (figure 30), thus suggesting that the amount of protein was not the limiting step.

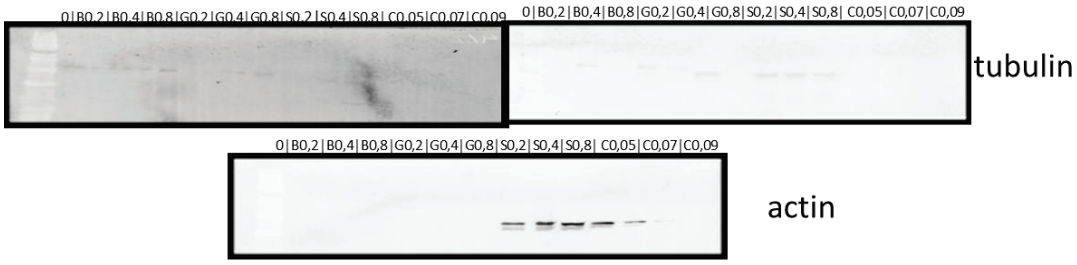


Figure 30: PVDF membrane showingimmunocomplexes due to tubulin and actin protein. All samples are from 144hpf larvae, and are the same in the three experiments. (Tubulin 30ug (right) and 70ug (left). Actin for 30ug of loaded protein .All three blots correspond to the same samples.

Considering that it was not possible to detect cytoskeleton proteins, it was posed the hypothesis that proteins were not being adequately denatured. Therefore, different denaturing buffers were tested without an improvement in the results, figure 31. TBS-T formulation was also tested, but no significant improvement was seen (figure 32).

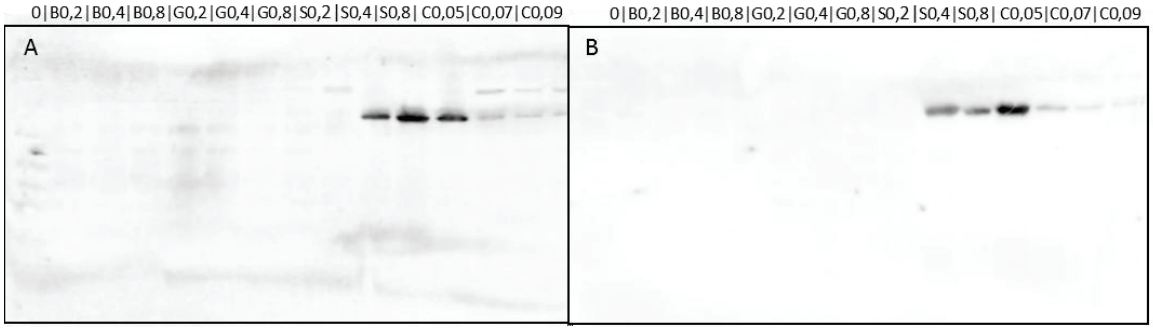


Figure 31: Actin labeling 1:1000. 30µg of protein from the same samples of 50hpf embryos used in both blots. Samples were denatured with two different buffers (A- denaturation buffer as indicated in materials and methods, B denaturation buffer without DTT and with B-mercaptoethanol)

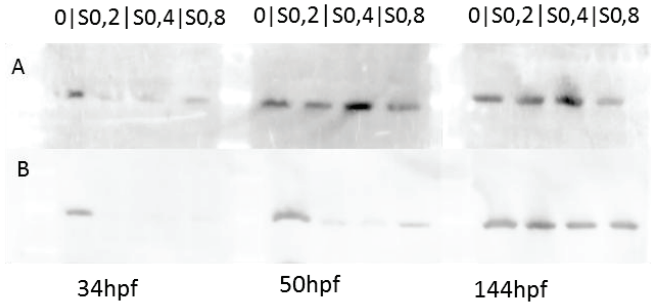


Figure 32: Samples from zebrafish exposed during segmentation and collected at 34, 50 and 144hpf. 30µg of protein was loaded. Probed for actin1:1000. A- TBS-T as indicated in materials and methods was used. B-TBS-t with 25mM Tris and 0,05% tween was used.

All the conditions tested did not improve western blot efficiency and we were not able to get any protein labeling in most of the samples. In the end, only samples obtained from segmentation stage were used. Degradation of proteins due to an inadequate storage and transportation is the most plausible explanation for such differences among samples of same development stages.

5.5.Troubleshooting associated to Fluorescence Microscopy

BrdU incorporation was detected using a cell proliferation ELISA kit, as already described. In order to identify the proliferation areas among CNS, we tried to detect BrdU through immunofluorescence and confocal microscopy. However, the autofluorescence associated to the fixative and also to the zebrafish made this task difficult. (figure 33) A few techniques to reduce autofluorescence will be tested in the future.

Troubleshooting

Due to autofluorescence it was not possible to achieve good results with the TUNEL assay (Figure 34). Furthermore, the attempt to evaluate alterations in the cytoskeleton using phalloidin to stain F-actin was also limited by autofluorescence (figure 35)

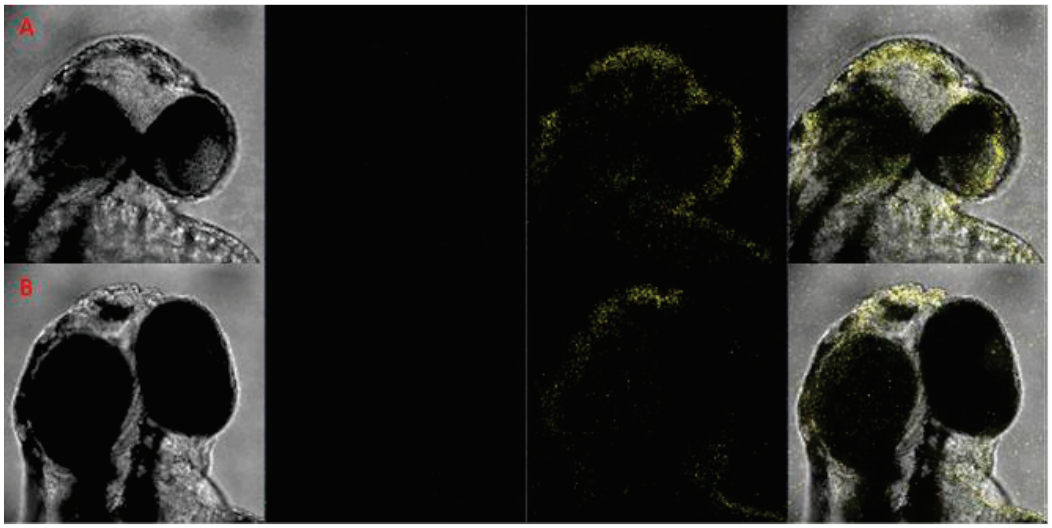


Figure 33:A- Embryos with BrdU (green) and Hoescht (yellow). B- Negative control (no BrdU) with Hoescht. Comparison between BrdU labeled and no BrdU labeled. Signal is due to autofluorescence. Magnification 400x.

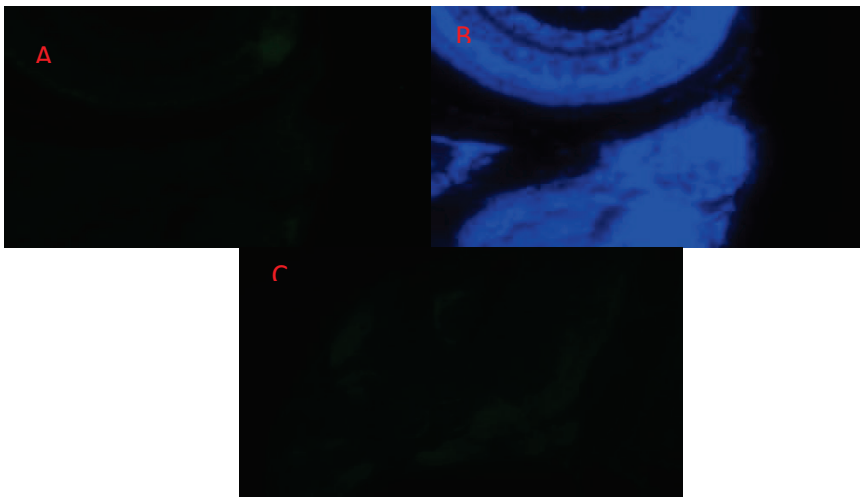


Figure 34: A: TUNEL assy; B:DAPI.C: negative control. The only signal observed is due to autofluorescence. No differences between negative control and other samples found. Control embryo, no ketamine treatment. Representative of all groups. Magnification 400x.

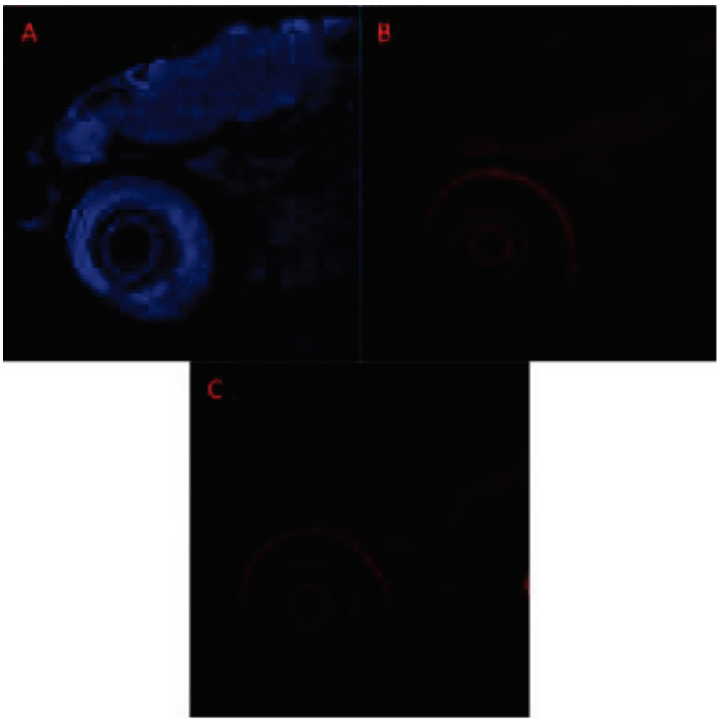


Figure 35: A: Phalloidin labeling; B-DAPI.C- negative control. No differences were found between the negative control and phalloidin labeled ones. No specific labeling is seen, only autofluorescence. Control group, no ketamine treated. Representative of all groups. Magnification 200x.

6.Discussion

Zebrafish anesthesia is frequently used in laboratory research, especially during procedures that require zebrafish immobilization. There are several anesthetics available, being the most common the MS-222. However, this anesthetic has several side effects that could interfere with the results in a given experiment. Therefore, it is of extreme importance to find an alternative anesthetic with a safety margin and reduced side effects.

Ketamine is used in anesthetic or subanesthetic doses to induce analgesia, amnesia, to suppress fear, anxiety and depression. In spite of being widely used, its molecular mechanism is controversial and seems to be dose-dependent. Previous studies pointed that when used in sub anesthetic doses ketamine could act as neuroprotective, and may stimulate synaptogenesis. However, when used in higher doses for a prolonged period of time it may be neurotoxic inducing neuronal damage. Furthermore, several studies pointed that when ketamine is used in the initial phases of CNS development it may decrease neural stem cell proliferation. On the other hand, a recent study using a human embryonic stem cell model found that ketamine increased neural stem cell proliferation without inducing apoptosis or neuronal damage (Bai et al., 2003). These controversies could be a consequence of the differences among the experimental models and therefore need to be better studied.

Considering that zebrafish embryos and larvae could be used as animal models in prolonged experiments and there are doubts regarding the effect of ketamine in their CNS development it is essential to evaluate the effect of ketamine at the different stages of development. Therefore, this study aimed to evaluate the cell proliferation, apoptosis and autophagy in tissue sections and cell lysates of Zebrafish embryos and larvae exposed to different concentrations of ketamine at different stages of development.

Due to several technical difficulties it was not possible to accomplish the study of apoptosis nor of autophagy. However, regarding the effect of ketamine in cell proliferation our results suggested that none of the ketamine concentrations used alter the cell proliferation. The results also showed that this effect was independent of the embryo development stage.

The study of the PCNA expression by immunohistochemistry also showed that the cell proliferation areas was restricted to specific areas that were similar to those previously identified by other groups: dorsal telencephalic proliferation zone, tectal proliferation zone and mesencephalic lamina in the mesencephalon, and cerebellum. (Grandel et al.,2006)

Considering that there were no alterations in the proliferative potential of CNS of zebrafish even when embryos were treated with the higher dosage of ketamine, it is important to determine by HPLC the concentration of ketamine in embryos and in the chorion at the end of the incubation period. The chorion is a barrier that slows down the diffusion of molecules (Ali et al.,2011) therefore, the delivery of ketamine may be

impaired, meaning that ketamine concentration in embryos could be less than initially planned.

In order to understand how zebrafish embryos were able to maintain the proliferative potential upon the treatment with ketamine, it is important to determine the expression of the ketamine targets at different stages of development. Considering that ketamine interacts mainly with NMDA-R and most of its effects are mediated through the interaction with this receptor, it is determinant to evaluate its expression and activity. In a previous study performed by Cox and colleagues, the NMDA-R expression was detected at 24 and 48h. (Cox et al.,2005). Therefore, it is expected that NMDA-Rs were not present during the blastula and gastrula stages which may protect these embryos from the neurotoxic effects of ketamine and may explain the maintenance of the cell proliferative ability during these two stages. However, considering the study performed by Felix et al. that hypothesized that in the zebrafish blastula stage, ketamine may exert its effects through an independent NMDA-R receptor it is important to study the expression of other receptors that may also interact with ketamine, such as AMPA-R. There is no data regarding the expression of this receptor during zebrafish embryo development but previous studies supported that upon AMPA-R stimulation, ketamine may promote synaptogenesis. This mechanism may also explain, the maintenance of the proliferative potential upon exposure of embryos and larvae to ketamine (Higashima et al., 2004; Boehmler et al., 2004; Schneider et al., 2012)

The greatest vulnerability of developing brain to anesthetics occurs at the time of rapid synaptogenesis. During this period both synapse formation and elimination occur asynchronously as concurrent processes. In addition multiple sequential steps are involved in neurogenesis including the proliferation of neural stem cells and its differentiation into neurons. Therefore, it is important to use neural stem cells markers such as Sox-2 to determine the location of these cells and also to determine if ketamine alters their pattern of distribution as well as their proliferation and survival ability.

Another point that should be considered is the effect of repetitive exposure to ketamine on the development of CNS. This question is particularly important since in most of the studies zebrafish need to be anesthetized at different days. The existence of a cumulative effect of ketamine should be investigated in order to establish the safety intervals as well as the safety dosages.

Furthermore, the results from this study put in evidence that there are technical details that should be carefully controlled, such as: an increased care with collection, storage and transportation of the samples, to avoid autolysis and ensure their quality; alternative histological fixation and processing such as high-throughput histology processing, to avoid the variability within sections (Sabaliauskas et al.,2006). The reducing of autofluorescence due to fixation and to extraembryonic structures is also a concern, the current protocol should therefore be altered.

Working with new model organisms in early stage of a project can be a major difficulty, because technique optimization can take a long time and may limit the acquisition of results in a short period of time. Also, animal models always present a

certain variability which can lead to different results, and so increasing sample number is important to dissipate this variability.

However, working with a new animal model is also challenging because it obliges to understand the details of each procedure and of the animal model characteristics. At the same time the development of a new project allows the establishment of collaborations between different research groups which is rather stimulating.

7.Conclusion

In conclusion, the main result of this study indicated that ketamine does not alter the proliferation potential of zebrafish embryo's CNS. This result could be a hallmark in the area of zebrafish research since it suggested that ketamine could be a good anesthetic. However, since the evaluation of apoptosis, autophagy were limited, further studies are needed to fully investigate the importance of ketamine in zebrafish anesthesia.

Furthermore, considering the results regarding cell proliferation, and the importance of ketamine in anesthesia it is important, in a near future, to evaluate the molecular mechanism associated to the maintenance of the cell proliferation and to understand the role of the neural stem cells to it.

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