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Longitudinal effects of ketamine on cell proliferation and death in the CNS of zebrafish

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ABSTRACT

Zebrafish is known for its widespread neurogenesis and regenerative capacity, as well as several biological advantages, which turned it into a relevant animal model in several areas of research, namely in toxicological studies. Ketamine is a well-known anesthetic used both in human as well as veterinary medicine, due to its safety, short duration and unique mode of action. However, ketamine administration is associated with neurotoxic effects and neuronal death, which renders its use on pediatric medicine problematic. Thus, the evaluation of ketamine effects administration at early stages of neurogenesis is of pivotal importance. The 1-41–4 somites stage of zebrafish embryo development corresponds to the beginning of segmentation and formation of neural tube. In this species, as well as in other vertebrates, longitudinal studies are scarce, and the evaluation of ketamine long-term effects in adults is poorly understood. This study aimed to assess the effects of ketamine administration at the 1–4 somites stage, both in subanesthetic and anesthetic concentrations, in brain cellular proliferation, pluripotency and death mechanisms in place during early and adult neurogenesis. For that purpose, embryos at the 1–4 somites stage (10.5 h post fertilization - hpf) were distributed into study groups and exposed for 20 min to ketamine concentrations at 0.2/0.8 mg/mL. Animals were grown until defined check points, namely 50 hpf, 144 hpf and 7 months adults. The assessment of the expression and distribution patterns of proliferating cell nuclear antigen (PCNA), of sex-determining region Y-box 2 (Sox 2), apoptosis-inducing factor (AIF) and microtubule-associated protein 1 light chain 3 (LC3) was performed by Western-blot and immunohistochemistry. The results evidenced the main alterations in 144 hpf larvae, namely in autophagy and in cellular proliferation at the highest concentration of ketamine (0.8 mg/mL). Nonetheless, in adults no significant alterations were seen, pointing to a return to a homeostatic stage. This study allowed clarifying some of the aspects pertaining the longitudinal effects of ketamine administration regarding the CNS capacity to proliferate and activate the appropriate cell death and repair mechanisms leading to homeostasis in zebrafish. Moreover, the results indicate that ketamine administration at 1–4 somites stage in the subanesthetic and anesthetic concentrations despite some transitory detrimental effects at 144 hpf, is long-term safe for CNS, which are newly and promising results in this research field.

1. Introduction

Zebrafish is a teleost species known for its robust and widespread brain neurogenesis, not only during development but also throughout adulthood, which allied to an extensive regenerative capacity after injury (Edelmann et al., 2013; Grandel et al., 2006) makes zebrafish a widely used model in several neurotoxicity studies (Cassar et al., 2020; Kanungo et al., 2013; Robinson et al., 2017). In addition to its small size, prolific reproductive capacity, and easy maintenance, zebrafish maintains the typical complexity of vertebrate systems, and accumulating

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evidence advocates the use of this species in several areas of research, namely toxicity, with the prospect of extrapolating findings to other vertebrates and humans (Cassar et al., 2020; Cuevas et al., 2013; Kanungo et al., 2013).

Ketamine is an anesthetic widely used both in human medicine as well as veterinary medicine (Bai et al., 2013; Cheung and Yew, 2019; Dinis-Oliveira, 2017). Its use on pediatric medicine may be problematic, as it is known that the developing brain is particularly susceptible to the neurotoxicity of ketamine, when compared to the mature adult brain (Cheung and Yew, 2019). Growing evidence suggests that the exposure of immature or developing brains to general anesthetics, namely ketamine, induces widespread neuronal cell death, followed by long-term memory and learning abnormalities due to permanent neurological brain injuries (Bai et al., 2013; Zhang et al., 2018). Additionally, multiple lines of evidence implicate ketamine in the alteration of neural stem cell differentiation (Akeju et al., 2014; Cuevas et al., 2013; Félix et al., 2014; Kanungo et al., 2013). Consequently, ketamine is presently one of the most studied anesthetics for addressing neurotoxicity issues in rodents and primate models (Bai et al., 2013) and recent studies have already established the teratogenicity of this anesthetic in zebrafish (Cuevas et al., 2013; Félix et al., 2014; Kanungo et al., 2013; Robinson et al., 2017).

It is well documented that ketamine's toxicity is associated with changes in caspases gene expression, interfering with the apoptotic processes during development in zebrafish (Félix et al., 2017; Kanungo et al., 2013). However, longitudinal studies are scarce, and the evaluation of the long-term effects in adults poorly studied.

The aim of this study was to evaluate the effects of subanesthetic, and anesthetic concentrations of ketamine administered at the 1–4 somites stage, in the central nervous system (CNS) of embryos (50 h post fertilization (hpf), larvae (144 hpf) and adults (7 months old) zebrafish. Pluripotency and proliferation capability was evaluated by studying the expression patterns Sox 2 and PCNA. To assess if the expected alterations parallels with the apoptotic and autophagic pathways, the expression of AIF and LC3 were also investigated.

2. Materials and methods

2.1. Chemicals

Ketamine (ketamine hydrochloride, Imalgene®1000, 100 mg/mL, purity >95%) was obtained from Merial Portuguesa-Saúde Animal Lda (Rio de Mouro, Portugal). All solutions were freshly made with embryo water (28 ± 0.5 °C, 200 mg/L Instant Ocean Salt and 100 mg/Lsodium bicarbonate (CAS 144–55–8); UV sterilized) prepared from City of Vila Real filtered-tap water. Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebourg, France).

2.2. Animals

Zebrafish (*Danio rerio*) wild-type (AB strain) maintenance and embryo collection were performed as described previously (Félix et al., 2016, 2017). Briefly, adult zebrafish were maintained in an open water system supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5) at 28 ± 0.5 °C in a 14:10 h light: dark cycle, at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). The fish were fed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia sp. nauplii*. Embryos were obtained after random mating of adult zebrafish, collected at 1 or 2 hpf, bleached and rinsed to remove debris, and then randomly distributed among treatment groups.

All experimental procedures were conducted in accordance with the licenses approved by the Institutional Animal Care Committee and by the Portuguese competent authority (Direção-Geral de Alimentação e Veterinária, Lisboa, Portugal) for the protection of animals used in research and in agreement with both the European (Directive 2010/63/EU) and Portuguese (Decreto-lei 113/2013) legislations on welfare of laboratory animals to minimize animal distress.

2.3. Ketamine exposure and sampling

Zebrafish embryos at 1–4 somites stage (10.5 hpf) (Kimmel et al., 1995) were exposed to ketamine concentrations (0.2 and 0.8 mg/mL, respectively 0.84 and 3.37 mM) during 20 min in 50 mL beakers. These concentrations were selected based on previous group work with ketamine, and range from sub-anesthetic (0.2 mg/mL) to anesthetic (0.8 mg/mL) ketamine concentration (Félix et al., 2016, 2017). At the end of the exposure period, embryos were rinsed three times with embryo water and allowed to develop at 28 ± 0.5 ºC until defined time periods (Fig. 1).

For western blot (WB), animals were allowed to develop until 50 hpf (50 embryos per replicate), 144 hpf (30 larvae per replicate) and 7 months old (2–3 brains per replicate). At least three independent replicates (4 for PCNA) were used. Animals were stored in PBS and kept at − 80 ºC until use.

For immunohistochemistry (IHC), animals developed until 144 hpf and 7 months, when they were analyzed. At these points, larvae and adult zebrafish brain were collected and fixed in 4% formaldehyde for 24 h and then kept in 70% ethanol at 4 ºC until use. A minimum of 5 up to 10 larvae were fixed and processed in order to optimise the process of animals' fixation in a lateral position, allowing to obtain brain sagittal sections.

All animals were euthanized by rapid cooling. Euthanasia of adult animals was followed by decapitation and brain dissection for WB or brain decalcification for IHC (see decalcification details below).

2.4. Western blot (WB)

The expression of Sox2, PCNA, LC3 and AIF were analysed by Western blotting as originally described by Balça-Silva et al. (2017). Briefly, embryos, larvae or adult brains were thaw immediately before use. Homogenates were centrifuged at 500g for 10 min at 4 °C. The supernatants were discarded. Then pellets were resuspended in RIPA buffer and then sonicated. After that, the samples were denatured with Laemmli buffer 2 × added to each sample at a 1:1 ratio. Protein extracts were boiled at 95 °C for 5 min before use. Thirty micrograms of protein were run on a 10% SDS-PAGE gel, transferred to a PVDF membrane, and then incubated with a solution of 5% non-fat milk in TBST for 1 h at room temperature. The primary antibodies against PCNA (PC10, Vector Labs®) at 1/100, SOX2 (ab 97959, 35795 Abcam®) at 1/10000, LC3 (#3848, Thermo Fisher®) at 1/10000, AIF (#4642, Cell Signalling®) at 1/250, and β-actin (clone C4, MAB 1501, Chemicon®) at 1/2500 diluted in TBST with 1% non-fat milk, and incubated at 4 ºC overnight. After the incubation period, the immunocomplexes were detected with appropriate anti-rabbit antibody (1/10000) or anti-mouse antibody (1/10000) (GE Healthcare®, UK) and conjugated with horseradish peroxidase. Bands were obtained after exposing the membranes to an X-ray film and analysed through densitometry scanning using a Chem-iDoc MP imaging system (BioRad®, Benicia, CA, USA). The protein expression was quantified by using the software ImageJ® 1.49 v (Wayne Rasband, National Institutes of Health, Bethesda, MD) with the expression of β-actin used as a loading control. Each experiment was repeated three times.

As both β-actin and Sox 2 antibody identify a band at 43 kDa, after the detection of β-actin, the membrane was stripped to detect Sox2. Briefly, stripping consists in 5 min washes with TBST, then milliQ water, then NaOH 0.2 M, milliQ water and finally blocking the membrane with milk.

2.5. Immunohistochemistry (IHC)

Prior to IHC, sequential longitudinal sagittal sections of 144 hpf

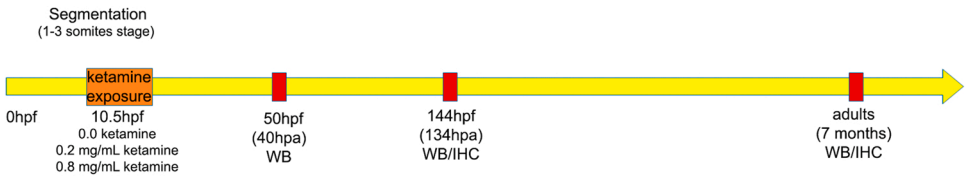

Fig. 1. Timeline of the study where animals were exposed at 10.5 h post-fertilization (1–4 somites) during 20 min to different ketamine concentrations (0.2 and 0.8 mg/mL). Animals were collected at 50 hpf, 144 hpf and 7 months for western blot (WB) and at 144 hpf and 7 months for immunohistochemistry (IHC) analysis. hpf- hours post-fertilization, hpa – hours post-anesthesia.

larvae and adult's zebrafish head were made and stained with haematoxylin-eosin (HE) to select the best section for IHC, i. e., the ones presenting all the major CNS subdivisions as in Grandel et al. (2006). The selection of the appropriate sections (aiming sagittal section 34) and anatomical description was done according to Wullimann et al., (1996) atlas, sagittal section 34 .

The IHC protocol was described in Santos and Pinto (2018) and in Balça-Silva et al. (2017). Shortly, whole larvae (144 hpf) and head samples (7 months) were fixated in 4% formaldehyde for 24 h, dehydrated, embedded in paraffin, and then sectioned (3-μm). A mild decalcification (for a few seconds) with 7% nitric acid was performed in adults, just prior to sectioning. Sections were deparaffinized in xylene and then rehydrated in an ethanol series followed by bath in tap running water. All sections were incubated for 30 min with 3% H_2O_2 (Sharlau®). Antigens were retrieved with citric acid buffer in a microwave (two or three cycles of 5 min, 400 W). After cooling, slides were washed with TBS-T (pH 7.6). Sections were then incubated in normal horse serum (Vector Laboratories®) at room temperature for 30 min (or blocking serum according to manufacturer's instructions), and further incubated with appropriate primary antibodies (anti-PCNA 1/100, anti-SOX2 1/1000, anti-AIF 1/200; anti-LC3 1/900) diluted in TBS overnight at 4 °C, except PCNA (1 h at room temperature). Sections were washed with TBS-Tween (pH 7.6) and incubated with the proper secondary antibody, biotinylated anti-polivalent solution or a non-biotin system such as ImmPress® universal antibody kit (Anti-mouse/rabbit Ig, cat. no. MP-7500, Vector Laboratories®) at room temperature. For each antibody a negative control was used by substituting the primary antibody with TBS. Sections were then incubated with diaminobenzidine (DAB, Sigma®) for a few seconds or minutes depending on the primary antibody used, counterstained with Harry's ® haematoxylin, dehydrated and mounted with DPX®. Each section was observed to attest the quality and intensity of the labelling of the positive cells as well as its distribution. All sections were observed with an Olympus CX 40 light microscope (Olympus®, Japan) and images acquired with the Dino-Eye Edge Eyepiece camera AM7025X, software DinoCapture 2.0 for windows (Dino-Lite Europe, The Netherlands).

2.6. Statistical analysis

All data are presented and analyzed as percentage of control that was normalized to 100%. Data of the treatment groups were compared verifying normal distribution with Shapiro Wilk test, and homogeneity of variances with Levene's test. For variables with normal distribution, the one sample T-test was used, comparing the values with the baseline 100%. When the data distribution was not normal, a two-step approach for transforming non-normally distributed variables in normally distributed was used (Templeton, 2011) and then the sample T-test was applied. If this transformation method did not achieve normality of data, the data were analyzed using the one-sample Wilcoxon signed rank test, comparing the values with the mean baseline, 100%. Statistical analyses were carried out using Graphpad® version 8.01 (GraphPad Software Inc., San Diego, CA, USA) software. Data were determined to be statistically significant if the p value was < 0.05.

3. Results

3.1. Proliferation

3.1.1. PCNA

Ketamine did not induce any alterations regarding the levels of PCNA in the 50 hpf embryos as demonstrated by the WB results (Fig. 2 A). Likewise, at 144 hpf, no significant alterations were observed between the control group and the group exposed to the ketamine subanaesthetic concentration of 0.2 mg/mL ($p = 0.0787$). However, a significant down-regulation in PCNA protein expression ($p = 0.0037$) was registered with the highest ketamine concentration (0.8 m g/mL) in 144 hpf larvae (Fig. 2, B I). In adults, the PCNA protein band of the expected size ca 0.29 kDa was not detectable by WB.

Nevertheless, PCNA immunoreactivity was observed in CNS sections in the defined time points. Representative images of the different study groups are shown in Fig. 2, B II (larvae) and C (adults). In larvae, immunoreactivity to PCNA was found in the CNS areas previously described to present high cellular proliferation, namely cerebellum (Fig. 2 B II), with apparent differences in the intensity of the immunopositivity. In line with the WB results for the 144 hpf larvae, these differences were observed between the control group and the highest ketamine concentration treatment group in the cerebellar area. Although it was not possible to detect PCNA in the WB of adults' brain, IHC technique showed that PCNA positive cells evidenced some variability among the different studied groups (Fig. 2 C). Some cells positive to PCNA with a neuroepithelial-like type morphology were seen in the cerebellum molecular layer (Fig. 2 C). These cells extended throughout the cerebellum molecular layer, above the Purkinje cells layer, towards the subpial area in an apparent migrating pattern (Fig. 2 C, control group, ketamine 0.2 mg/mL group). In the cerebellum sections from control group and ketamine 0.8 mg/mL group, some PCNA expressing cells presented similar features but those were observed more internally, just above the Purkinje cell layer; cells displaying a rounder phenotype were also observed in the molecular layer of the cerebellum (Fig. 2 C, ketamine 0.8 mg/mL group). It is noteworthy that sections of the control group and those exposed to the lowest ketamine concentration presented less/no round phenotype PCNA positive cells when compared to the group with the highest ketamine concentration (Fig. 2 C).

3.1.2. Sox 2

Regarding the expression of Sox 2 in the 50 hpf embryos, 144 hpf larvae and adults, despite some minor variations, there were no significant alterations (Fig. 3 A, B I and C I respectively) between the ketamine treatment groups and the baseline value of the control group. In larvae the immunoexpression of Sox 2 was evident, not only in the CNS, but also in the eye, intestine, and liver (Fig. 3 B II). In adults, the areas of the brain presenting Sox 2 immunoexpression were as expected (telencephalic ventricle, diencephalic ventricle, optic tectum, dorsal zone of periventricular hypothalamus and cerebellum). Considering the overall results of IHC, the adult cerebellum (Fig. 3 C II) was the selected area to exhibit representative images of the Sox 2 immunoexpression of the different groups, since is a proliferative area, which also presented cells expressing PCNA. Here, encephalic sections exhibited immunolabeling in the cerebellum molecular layer, presenting positive cells with round

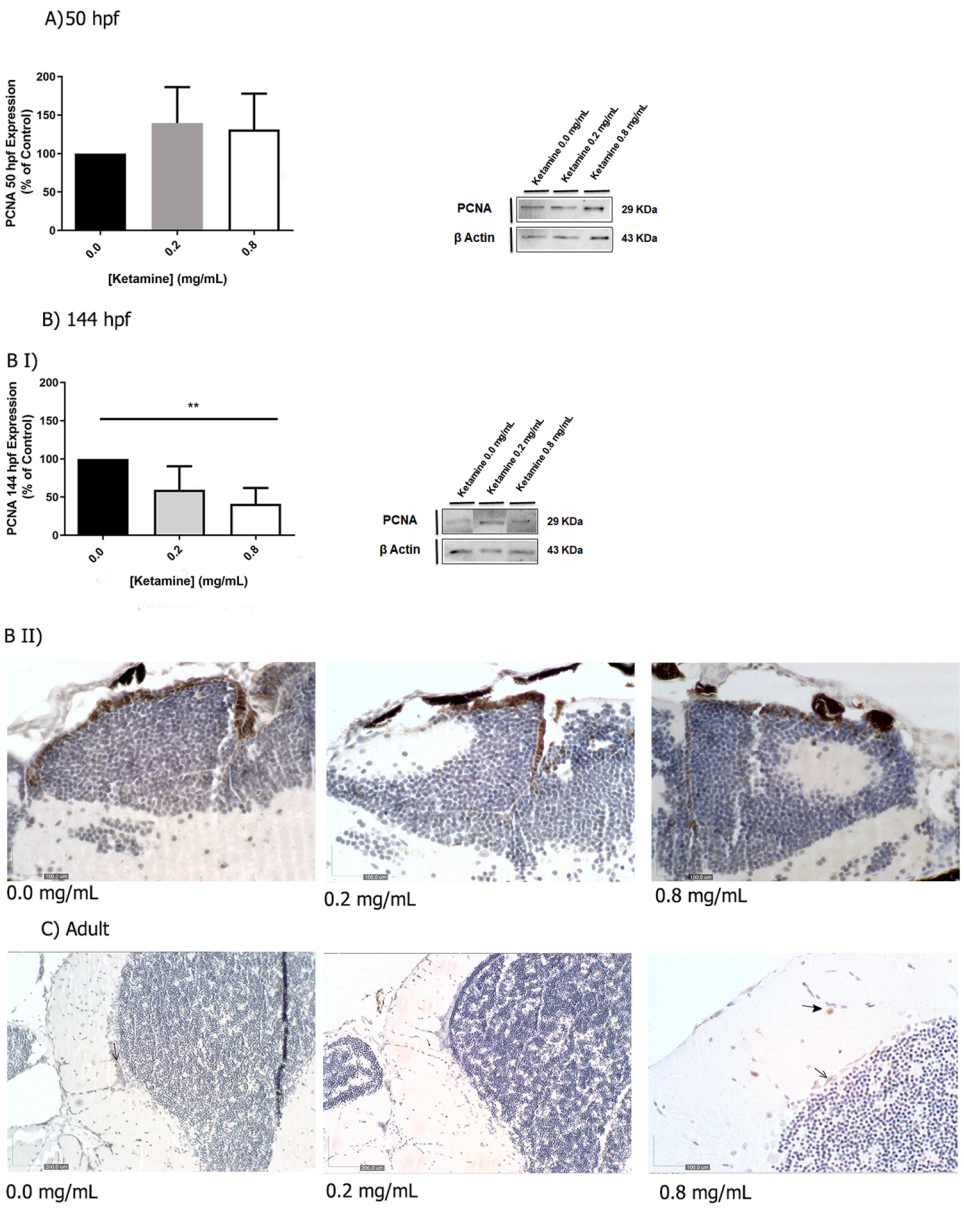

Fig. 2. The expression of PCNA in 50 hpf (**A**), 144 hpf (**B**) and in adult (**C**) zebrafish after being exposed to different concentrations of ketamine during 20 min at 1–4 somites stage. The levels of PCNA were quantified by western blot (**A,B I**), and data represented by mean + SEM of the percentage of control of four independent replicates; * * p = 0,0037 for comparison between the group ketamine 0.8 mg/mL and the baseline value 100 using one sample T-test after data normalization (**B I**). The expression patterns of PCNA were observed by immunohistochemistry (**B II**, **C**). Immunoreactivity to PCNA was found in the CNS areas previously described as presenting high cellular proliferation, such as cerebellum (**B II**). Representative images of adult brains with focus on the cerebellum (**C**) reveal two types of positive cells, one with a neuroepithelial-like morphology in cerebellum molecular layer (arrow) present in all groups and others with a round morphology observed in the molecular layer of animals exposed to ketamine 0.8 mg/mL group (solid arrow). Scale bar 100 μm (**B II,** and **C-** 0.8 mg/mL ketamine group) and 200 μm (**C-** control and 0.2 mg/mL ketamine group).

nuclei. Animals treated with the highest ketamine concentration exhibited immunopositivity in cells with round morphology in the molecular layer, but also evidenced immunopositivity in the Purkinje cells, at the Purkinje cells layer (Fig. 3 C II). Purkinje cell layer immunoexpression was seen in animals exposed to the highest ketamine concentration being absent in the other study groups.

3.2. Cellular death

To further assess if the potential alterations in proliferation in CNS zebrafish embryos, larvae and adults were related with the apoptotic and autophagic pathways, AIF and LC3 expression were evaluated, respectively.

3.2.1. Apoptotic effects - AIF

In the 50 hpf embryos, WB results revealed no significant alterations in the AIF levels of the ketamine exposed groups compared with the baseline. However, the AIF levels seem to increase in the group exposed to the highest ketamine concentration (0.8 mg/mL group) (Fig. 4 A). The AIF levels in the 144 hpf larvae or adult brains only had slight alterations (Fig. 4 B I and C I respectively). Likewise, no visible alterations were observed in the distribution or immunoexpression patterns of the CNS positive areas to AIF in 144 hpf larvae or adult zebrafish (Fig. 4 B II and C II).

3.2.2. Autophagic effects - LC3

Considering LC3 WB results, no apparent alterations of LC3 levels were observed in the 50 hpf embryos (Fig. 5 A), or in the adult brain (Fig. 5 C I). However, the 144 hpf larvae exposed to 0.8 mg/mL of ketamine had a significant decrease in the LC3 levels (p = 0,0404) (Fig. 5 B I). On the contrary, an up-regulation of the LC3 levels in the 0.2 mg/mL group seemed to occur, although not significantly different from the control group (p = 0.0579). However, these differences were not visible in the IHC results for LC3 (Fig. 5 B II), and, also, its expression pattern did not seem different between groups in the CNS of adult zebrafish (Fig. 5 C II).

4. Discussion

Ketamine remains a widely used anesthetic, in both human and

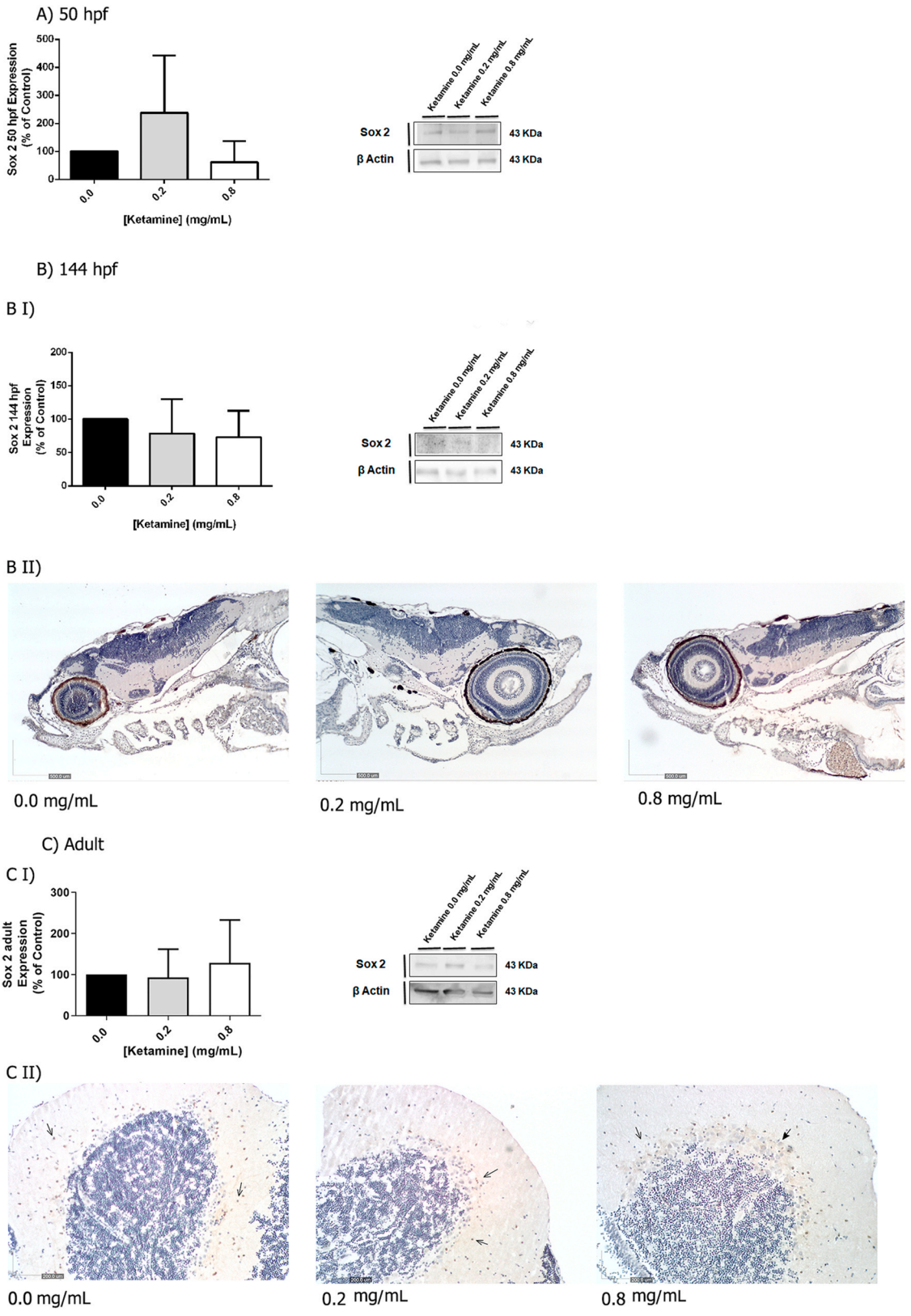

Fig. 3. The expression of Sox 2 in 50hpf (**A**), 144hpf (**B**) and in adult (**C**) zebrafish after being exposed to different concentrations of ketamine during 20 min at 1–4 somites stage. The levels of Sox 2 were quantified by western blot (**A**, **B I** and **C I**), and data represented by mean + SEM of the percentage of control of three independent replicates. The cells exhibiting Sox 2 were observed by immunohistochemistry (**B II**, **C II**). **B II** represents Sox 2 in larvae evidencing immunolabeling in CNS and other organs. Representative images of adult brains with focus on the cerebellum (**C II**) reveal two types of positive cells, one in the cerebellum molecular layer (open arrow) represented in all groups, and the other in the Purkinje cells in the cerebellum Purkinje cells layer exhibited by the highest ketamine concentration (solid arrow). Scale bar 500 μm (**B II**) and 200 μm (**CII**).

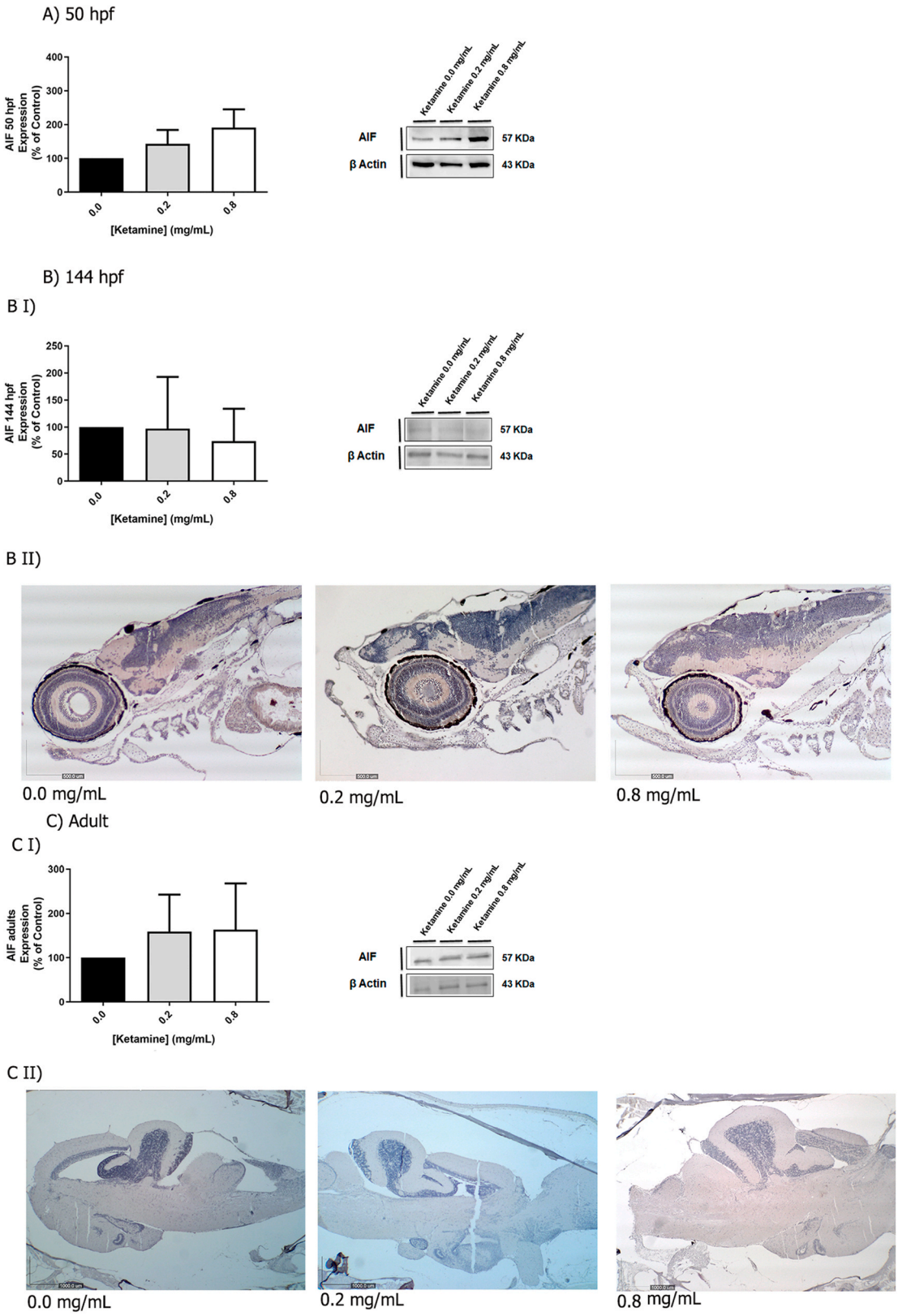

Fig. 4. The expression of AIF in 50hpf (**A**), 144hpf (**B**) and in adult (**C**) zebrafish after being exposed to different concentrations of ketamine during 20 min at 1–4 somites stage. The levels of AIF were quantified by western blot (**A**, **B I** and **C I**), and data represented by mean + SEM of the percentage of control of three independent replicates. The areas exhibiting AIF were observed by immunohistochemistry (**B II**, **C II**). **B II** represents AIF in larvae evidencing immunolabeling in CNS. Representative images of adult brains with focus on cerebellum (**C II**) also presented CNS immunolabeling with no visible alteration. Scale bar 500 μm (**B II**) and 200 μm (**CII**).

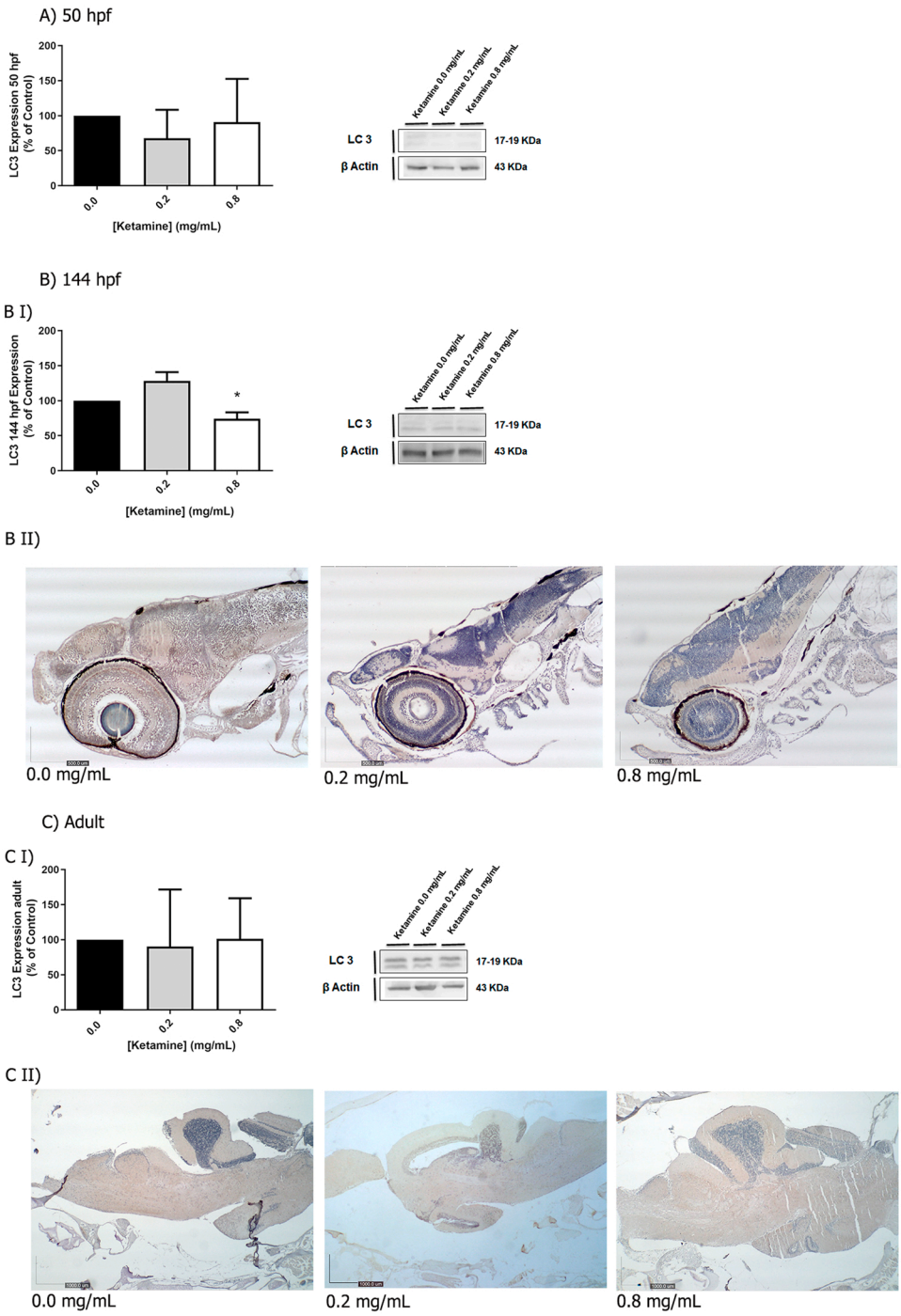

Fig. 5. The expression of LC3 in 50hpf (**A**), 144hpf (**B**) and in adult (**C**) zebrafish after being exposed to different concentrations of ketamine during 20 min at 1–4 somites stage. The levels of LC3 were quantified by western blot (**A**, **B I** and **C I**), and data represented by mean + SEM of the percentage of control of three independent replicates; *p = 0,0404 for comparison between the group ketamine 0.8 mg/mL and the baseline value 100 using one sample T-test. (**B I**). The IHC results for LC3 in 144 hpf larvae (B II) and in adult brains (C II) evidence immunolabeling in CNS, with no visible alterations. Scale bar 500 μm (B II) and 1000 μm (C II).

veterinary medicine, due to its safety, short duration and unique mode of action, being important in emergency medicine and pain management around the world (Kohtala, 2021). However, given the known effects of anesthetics on the developing brain, it is important to assess ketamine neuroteratogenicity; zebrafish was used as a relevant model to assess toxicity effects. The rationale was to test two different physiological states (depths of anesthesia) to have a broad view of ketamine effects. Therefore, the present study aimed to describe the longitudinal effects of sub-anesthetic and anesthetic concentrations of ketamine when administered at the 1–4 somites stage of zebrafish, evaluating brain cellular proliferation and cellular death. The 1–4 somites stage correspond to the beginning of segmentation stage, where the formation of neural tube takes place originating the CNS (Kimmel et al., 1995), highlighting the importance of evaluating the effects of ketamine administration on the brain pluripotency and proliferation capacity at this developmental stage. Pluripotency, apoptosis, and phagocytic pathways were assessed by investigating the expression and distribution of PCNA, Sox 2, AIF and LC3 by western blot and immunohistochemistry in the CNS, respectively. Overall, the highest concentration of ketamine (0.8 mg/mL) decreased autophagy assessed by LC3, and cellular proliferation assessed by PCNA at 144hpf. Our study employed a ketamine concentration (3.37 mM) slightly higher than the lowest value detected in human blood (2 mM). Therefore, our findings could also be particularly valuable for developmental research, as fetal blood often presents higher levels of ketamine compared to maternal blood (Félix et al., 2019; Robinson et al., 2016).

4.1. PCNA

A significant down-regulation of PCNA levels, and subsequently decrease in CNS proliferation, were observed in zebrafish larvae (144 hpf) exposed to the highest ketamine concentration, however, no significant alterations were observed in 50 hpf embryos or adults. As regards IHC immunoexpression, results were similar to the described in other studies (Grandel et al., 2006; Kenney et al., 2021) revealing the same CNS proliferative areas, such as telencephalon, diencephalon, optic tectum and the cerebellum, in 144 hpf larvae and adults. Moreover, intensity differences of PCNA immunoexpressing cells in larvae brain were similar to the WB results at the same timepoint, with sections of the highest ketamine concentration presenting less intensity in the immunopositivity when compared to the control group sections. In addition, in adults, the morphology of some positive cells showed a neuroepithelial-like type in the cerebellum molecular layer, as observed by Kaslin et al., (2013, 2017). In the lowest ketamine concentration group, those cells extended throughout the molecular layer. However, in control and the highest concentration groups, neuroepithelial-like cells were observed more internally, just above the Purkinje cell layer. Those observations indicate that despite those cells maintained a cycling capacity, they presented different migrating patterns, or migration at different pace across the cerebellum molecular layer. Kaslin et al., (2017) and Lindsey et al., (2019) described that the population of neuroepithelial-like stem cells remained stable with age in the cerebellum, while other populations become quiescent or exhaust. It is also known that in adults' cerebellum not all cell types are produced after an injury (Kaslin et al., 2017). Thus, concerning the cells with the round nuclei observed at the molecular layer of the highest ketamine concentration group, further studies will be needed to confirm their phenotype as differentiated neurons or other cell type.

Therefore, ketamine may not only alter the expression pattern of PCNA but the migration/differentiation time of the cerebellar cell types (neuroepithelial like or others) in adults, contributing to a general alteration of CNS cellular proliferation.

These results of altered proliferation after high concentration of ketamine administration are in accordance with other studies, which refer to alterations in neuronal differentiation (Kanungo et al., 2013) and inhibition of neuronal proliferation in rat fetal cortex neurogenic regions following ketamine exposure in embryogenesis (Dong et al., 2016). Still, other study with a similar protocol to ours detected no relevant changes on the genetic expression of PCNA at 24 hpf (Félix et al., 2017). Indeed, our study just showed differences in the proliferation at 144 hpf, while no differences were observed earlier (50 hpf). It seems thus, that cellular mechanisms such as cell death programs, were at play following ketamine administration, but then, it may not be enough to correct all the accumulated cellular alterations induced by ketamine, leading to an impact in the proliferative mechanism. Knowing the relationship between cell proliferation and cell death processes (Loftus et al., 2022), and the cross-talk between those mechanisms (Loane et al., 2015), the evidence of alterations in cell proliferation and PCNA expression were delayed. In agreement with the knowledge that PCNA, considered a regulator of the cell cycle, may be crucial in influencing the cellular choice between different pathways in order to maintain genomic and cell stability (Ju et al., 2000; Zhu et al., 2014).

Nevertheless, failure to quantify PCNA by WB in brain homogenates from 7 months adults raised some questions about cell proliferation in adults. Although the nature of the insult is different, Kishimoto et al. (2012) indicated that after a lesion, the PCNA results returned to the baseline by 21 days post-lesion. Furthermore, both Edelmann et al. (2013) and Barbosa et al. (2015) described that the number of neural stem cells (NSC) significantly decreases in 6- and 10-months old zebrafish when compared to the number of NSC in 3-month-old fish, indicating that there is a decline in neurogenesis with age, and consequently a reduction of cycling cells. In addition, Obermann et al. (2019) reported a slow decrease in the mitotic activity of NSC with aging, both in mammals and zebrafish. It has also been demonstrated that, not only the NSC decreases, but the proportion of radial glia cycling also decreased significantly between 6 months and 2 years of age, i.e., the pool of neural stem and progenitor cells is gradually exhausted, which impacts the neural regeneration, especially in adults (Kaslin et al., 2017). Thus, despite the observation of some cycling cells in brains from 7 months old zebrafish by IHC, the lack of PCNA detection by WB is in agreement with the literature findings, pointing to a decrease in the population of NSC and cycling cells in CNS of zebrafish with more than 6 months old, rendering quantification more challenging.

To eliminate the possibility that factors such as batch variation or quality of the protein extracts could interfere with failure to detect PCNA in adult brains by WB, several repetitions of the protocol were made, with optimizations, and the results were consistently the same. Additionally, to corroborate the quality of the protein extract, the brain homogenates used were the same to all the studied proteins, with good results in all other studied proteins. Therefore, the findings seem, in fact, to be associated with the diminished PCNA expression in the CNS of animals older than 6 months. Exhaustion of neural stem and progenitor cells over time might be a general trait of vertebrate brains, namely of zebrafish (Barbosa et al., 2015), in agreement with the studies of Edelmann et al. (2013) and Kaslin et al. (2017).

4.2. Sox 2

Given the established Sox 2 functions, and its importance in stemness and proliferation (Ito et al., 2010; März et al., 2010), the expression and quantification of this protein was also evaluated, but no significant alterations were detected after ketamine exposure, indicating that the functions associated with Sox 2 were not disturbed in any developmental stage observed.

Sox 2 expression was observed in the CNS, as expected, and nothing relevant was observed in larvae IHC results. Regarding IHC results of adult brains, cells expressing Sox 2 in the cerebellum were different than those expressing PCNA. Adult animals exposed to the highest ketamine concentration exhibited Sox 2 immunolabeling in the Purkinje cells layer of the cerebellum; however, Sox 2 immunoexpression was not observed in this cerebellar layer in sections from the other groups. Despite this, our results seem to indicate that ketamine administration at the 1–4 somites stage has no apparent effects in the stemness of the CNS cell population, or other Sox 2 functions, in embryos, larvae or adults, contrarily to the results obtained for PCNA in larvae. Thus, it appears that ketamine did not interfere with the CNS stemness, regardless the observed altered cycling capacity at 144 hpf after ketamine exposure at 1–4 somites stage.

Moreover, the differences between Sox 2 and PCNA expression highlight the fact that Sox 2 has several different functions in CNS and is not directly responsible for proliferation. Although it is involved in regulating the balance between self-renewal and differentiation in stem cells, affecting proliferation, Sox 2 is present not only in stem cells but also in glial and differentiated cells, which made this protein quantifiable in adult brains, contrarily to PCNA (DeOliveira-Mello et al., 2019).

To further evaluate if the obtained results in proliferation and stemness capability parallels with alterations in the mechanisms of cellular death and repair, the expression and quantification of AIF and LC3 were assessed, at the same defined timepoints.

4.3. AIF

It is already established that ketamine administration is associated with apoptosis (Félix et al., 2017; Lundberg et al., 2020; Mak et al., 2010) yet the caspase dependent pathway is the most thoroughly studied. Alternatively, this study focused on the alterations seen in the AIF expression and distribution. AIF, in contrast to caspases, is a ubiquitously expressed flavoprotein with a critical role in caspase-independent apoptosis (Cheng et al., 2012) which, upon an apoptotic insult,

undergoes proteolysis and translocate to the nucleus, triggering chromatin condensation and large-scale DNA degradation. For these reasons AIF has emerged as a protein critical for cell survival, that has pro-survival and pro-death effectors through several interactions with the cytoplasmic proteins (Cregan et al., 2004; Sevrioukova, 2011).

In the present study, the AIF levels presented some variations, and, although the highest ketamine concentration upregulated the AIF levels at 50 hpf, this is not statistically significant, and no significant differences were detected in ketamine groups related to baseline. The WB results refer to the 57 kDa form of AIF, which is present after pathological permeabilization of the outer mitochondrial membrane and nuclear translocation (Sevrioukova, 2011). These results are in accordance with the findings of Félix et al. (2017), that screened several apoptotic genes after ketamine exposure at the 1–4 somites stage, and minor differences were detected between control and ketamine groups. Our group previously observed that, at 24 hpf, the expressions of *casp8* and *aifm1* genes were affected; *aifm1* transcript levels were increased, although in the low ketamine concentrations (Félix et al., 2017). At 1–4 somites stage, both the mechanism of apoptosis and of cell proliferation are fully functional (Félix et al., 2017; Ikegami et al., 1999), thus the apoptotic pathways, as expected, were the first to exhibit alterations in embryos stage. However, these alterations seemed transitory, as we did not find significant variations between study groups in 50 hpf embryos, in larvae or adults. Considering the role of the compensatory cellular repair mechanisms, and the cross-talk between different apoptotic pathways, it seems that in embryos, the selection of the apoptotic pathway depends on the condition of the cell, in agreement with the described by Loane et al. (2015), for example the cell stage. On the other hand, Bai et al. (2013) described that in human neural stem cells, ketamine enhances proliferation and induces neuronal apoptosis via reactive oxygen species (ROS) mediated mitochondrial pathway. This is supported by Polster (2013) review implicating AIF in diverse but intertwined processes, such as electron transport chain function, ROS regulation and neurodegeneration. As in the AIF analysis, the proliferation marker PCNA had its levels increased (not significantly) by ketamine at 50 hpf, which could indicate a shift to other mechanisms to protect the cells against ketamine exposure.

Similarly, no apparent differences were observed in AIF immunoexpression among the different study groups in larvae or adults. In adults, not all the groups presented evident immunoexpression in the brain, but other areas such as branchia presented clear positive cells.

Taking in consideration the literature referred and our results, at later stages, it is fair to assume that mechanisms other than apoptosis are selected to help the regulation of cellular processes after ketamine exposure and the return to baseline, such as the autophagy process studied here using the marker LC3.

4.4. LC3

It is well described that most tissues maintain a basal autophagic flux under normal physiological conditions (Corcelle et al., 2009; Zschocke et al., 2011) and autophagic activity might result in type II cell death, a process different from caspase-mediated apoptosis (Gozuacik and Kimchi, 2004; Zschocke et al., 2011). Besides, it is well documented that the autophagic process is a major mechanism of cellular constituents' turnover, and critical for cellular homeostasis (Boya et al., 2018; Lundberg et al., 2020; Rosenfeldt and Ryan, 2011). Indeed, autophagy is a phylogenetically conserved membrane trafficking process, delivering cytoplasmic cargoes to lysosomes for digestion (Rosenfeldt and Ryan, 2011) and so important to track.

Given the existing evidence in literature indicating LC3 as a biomarker for autophagy and functioning as a structural component during autophagosome formation (Lundberg et al., 2020; Martinet et al., 2006; Suzuki et al., 2015), the levels of this protein were assessed.

In the present study, no apparent alterations of LC3 levels were observed after ketamine exposure, in 50 hpf embryos or adults brain homogenates. Thus, it seems that the onset mechanism of cellular response to ketamine exposure is apoptosis, as referred in the literature (Félix et al., 2017) in embryos. However, in 144 hpf larvae a downregulation of the LC3 levels was observed in the highest ketamine concentration group indicating that, at this stage, ketamine administration altered not only proliferation, but also the autophagic process. Under stressful conditions, autophagy serves as a cell protective mechanism, preventing the accumulation of cytotoxic protein aggregates or damaged organelles (Satoh et al., 2014). As such, the impairment in the autophagic process may lead to increased cell death (Galluzzi et al., 2012). The alteration observed at 144 hpf could also be related with the increased ROS formation induced by the ketamine administration in embryos during 1–4 somites stage as described by Félix et al. (2016). In fact, several other studies have already linked ketamine-induced neurotoxicity to high levels of ROS (Choudhury et al., 2021).

Additionally, Li et al. (2018) have related higher ROS levels with the formation and degradation of autophagosomes, LC3 accumulation and then autophagy. However, in the present study, although LC3 levels were decreased by the highest ketamine concentration, the lowest ketamine concentration induced a non-significant increase in the LC3 levels in the 144 hpf larvae. Thus, the influence of ketamine on autophagy may be dependent of the concentration. Indeed, frequent administrations of ketamine downregulated the expression of autophagic proteins, such as LC3, in rats, probably through mTOR signalling pathway activation (Wang et al., 2017). Indeed, ketamine has been described to activate mTOR signalling in mammals (Choudhury et al., 2021), which is a key regulator of cell growth and proliferation. Therefore, it is hypothesized that the LC3 and PCNA downregulation occurring at the same time (144 hpf) in the present study could be related to the influence of ketamine in the mTOR signalling pathways, but further studies are needed to clarify this issue. Despite this, no apparent alterations between treatment groups and control were seen in the intensity of LC3 immunoexpression in CNS both in larvae and adults.

5. Conclusion

The main observed alterations following ketamine exposure at the 1–4 somites stage indicate that the highest ketamine concentration used (0.8 mg/mL) equivalent to 3.33 mM, impaired the autophagic process and cell proliferation in 144 hpf larvae. These findings confirm ketamine disturbed neuronal proliferation and autophagy, as registered in other species (Peters et al., 2018; Wang et al., 2017), and are similar to the described in humans where ketamine capacity to promote cell survival or death depends on its dose (Choudhury et al., 2021). Changes observed in this study were dose-dependent, and transitory since the studied proteins presented no significant variations in adult animals, indicating the return to a baseline. Thus, this study allowed to verify the longitudinal effects of ketamine administration in 1–4 somites stage regarding the CNS zebrafish capacity to proliferate and activate the appropriate cell death and repair mechanism leading to homeostasis.

The obtained results indicate that ketamine administration in zebrafish in 1–4 somites stage in the sub-anesthetic and anesthetic concentrations is long-term safe for CNS, although the migration/differentiation time of the cerebellar cell types or other features not studied may be affected. Nevertheless, the lack of differences in adult brain analysis is in agreement with the described capability of zebrafish to repair and regenerate injured organs, in this case the CNS (Kishimoto et al., 2012; Schmidt et al., 2013). Some of the deleterious effects of ketamine were countered by the cellular mechanisms of cell repair. Since in the adult zebrafish brain transcription factors homologous to the mammalian counterparts are at play in the neurogenic process (Obermann et al., 2019), zebrafish is a valid comparative vertebrate model organism. However, the genes and pathways involved in the initiation and maintenance of such an extensive regenerative response are largely unknown (Cosacak et al., 2015). Many parameters, like the age, the species, the type and size of the injury and the affected brain

area determine to which extent regeneration occurs in the teleost brain (Kizil et al., 2012). The knowledge of the mechanisms responsible for brain repair and regeneration are of paramount importance to better comprehend how we can activate those same programs in mammalian brains to achieve functional recovery (Cosacak et al., 2015), and mTOR signalling pathway is a potential target of interest to research (Lu et al., 2022). Hence, further studies are needed to better understand the underlying mechanisms of brain repair and regeneration, either after an insult (mechanical or chemical) or during the normal ageing process.

CRediT authorship contribution statement

C. Santos: Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Writing – review & editing. **A.M. Valentim:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **L. Félix:** Methodology, Writing – review & editing. **J. Balça-Silva:** Methodology, Validation, Data curation, Writing – review & editing. **M.L. Pinto:** Conceptualization, Methodology, Validation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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