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ORIGINAL INVESTIGATION

Behavioral alterations of zebrafish larvae after early embryonic exposure to ketamine

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Abstract
Rationale Ketamine has been associated with pediatric risks that include neurocognitive impairment and long-term behavioral disorders. However, the neurobehavioral effects of ketamine exposure in early development remain uncertain.
Objectives This study aimed to test stage- and dose-dependent effects of ketamine exposure on certain brain functions by evaluating alterations in locomotion, anxiety-like and avoidance behaviors, as well as socialization.
Methods Embryos were exposed to different concentrations of ketamine (0, 0.2, 0.4, and 0.8 mg mL^{-1}) for 20 min during the 256-cell (2.5 h post fertilization—hpf), 50% epiboly (5.5 hpf), and 1–4 somites (10.5 hpf) stages. General exploratory activities, natural escape-like responses, and social interactions were analyzed under continuous light or under a moving light stimulus.
Results A dose-dependent decrease in the overall mean speed was perceived in the embryos exposed during the 256-cell stage. These results were related to previously observed head and eye malformations, following ketamine exposure at this stage and may indicate possible neurobehavioral disorders when ketamine exposure is performed at this stage. Results also showed that ketamine exposure during the 50% epiboly and 1–4 somites stages induced a significant increment of the anxiety-like behavior and a decrease in avoidance behavior in all exposed groups.
Conclusions Overall, the results validate the neurodevelopmental risks of early-life exposure to ketamine.

Keywords Zebrafish larvae · Developmental stages · Locomotion · Ketamine · Anxiety-like behaviors · Avoidance response

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Introduction

Ketamine is a dissociative anesthetic agent, classified as an N-methyl-D-aspartate (NMDA) receptor antagonist, approved for human and veterinary medicines (Rofael and Abdel-Rahman 2002). Due to its unique pharmacological characteristics, ketamine is widely used in clinical practice (Kurdi et al. 2014; Morgan et al. 2012), with applications in both pediatric and obstetric anesthesia (Dong and Anand 2013). Despite its clinical applications, there were safety concerns about the hallucinogenic and dissociative effects of ketamine (Morgan et al. 2012). Indeed, a recent discussion about the safety of ketamine in the developing brain has been triggered since studies have described its potential to induce neurodevelopmental problems (Dong and Anand 2013; Su et al. 2010; Yan et al. 2014). Moreover, other studies have also linked ketamine anesthesia with potential long-term neurocognitive impairment in young children (Mellon et al. 2007; Wilder et al. 2009) and the prevalence of long-term behavioral disorders have been associated with early fetal exposure to anesthetics (Palanisamy 2012; Sprung et al. 2009).

These findings were further supported by several animal experiments suggesting that early brain development is

affected by ketamine exposure, resulting in behavioral disorders and neurodegeneration (Brambrink et al. 2012; Ikonomidou et al. 1999; Paule et al. 2011; Scallet et al. 2004; Viberg et al. 2008). Although it is commonly accepted that these responses are mediated by the NMDA receptor, the potential targets for ketamine also include biochemical signaling pathways independent of the glutamate receptor (Mion and Villevieille 2013). In this sense, much of the mechanisms of neurobehavioral effects that may result from early life exposure to ketamine remain unclear.

Recently, zebrafish (*Danio rerio*) has emerged as a complementary vertebrate model for neurobehavioral studies (Kalueff et al. 2013), especially during development. During the first week of development, zebrafish larvae can display complex and robust behaviors (Stewart et al. 2014). Although zebrafish behavior does not perfectly resemble mammalian responses, from a pharmacological perspective, this model shares similar neuroendocrine properties (Kalueff et al. 2013; Kalueff et al. 2014).

Previous studies have reported behavioral effects of ketamine in zebrafish embryo-larval stages (Burgess and Granato 2007; Suen et al. 2013; Wolman et al. 2011) and the induction of long-term malformations after exposure during the 256-cell period (Felix et al. 2014). Still, very early exposure to ketamine and its long-term implications on behavior have not been assessed. Thus, the goal of the present study was to evaluate potential defective brain functions revealed by alterations on locomotion, anxiety-like, avoidance and social behaviors, following ketamine exposure during zebrafish early developmental stages (256-cell, 50% epiboly, and 1–4 somites stage).

Material and methods

Statement of ethic on animal use

All procedures were conducted under personal and project licenses for this study in agreement with European Directive on the protection of animals used for scientific purposes (2010/63/EU) and its transposition to the Portuguese law (Decreto-lei 113/2013), ensuring minimal animal stress and discomfort. The experiments performed in this work were under project license approval by the Portuguese Competent Authority (Direção-Geral de Alimentação e Veterinária).

Fish husbandry and eggs production

Zebrafish maintenance and embryo collection were performed has previously described (Felix et al. 2014). Briefly, adult zebrafish from AB strain were kept at a maximum density of 40 animals in a 20-L glass aquaria and maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) 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 on a 14:10 h light: dark cycle (lights on at 8:00 am). The fish were fed twice daily with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. nauplii. Eggs were obtained by random pairwise mating of adult males and females (ratio of 2:1) in the evening before spawning induction. The eggs were harvested in the following morning within 1 h after spawning. Eggs were washed out three times with embryo water, bleached according to established protocols (Varga 2011; Westerfield 2007) and rinsed to remove debris. Unfertilized, unhealthy and dead embryos were removed and embryos with normal morphology were staged under an SMZ 445 stereomicroscope (Nikon, Japan) according to standard methods (Kimmel et al. 1995).

Chemicals

Ketamine (ketamine hydrochloride, Imalgene1000, 100 mg mL^{-1}) 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^{-1} Instant Ocean Salt and 100 mg L^{-1} sodium bicarbonate; UV sterilized) prepared from City of Vila Real filtered-tap water. Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebourg, France), while agarose was purchased from NZYTech (Lisboa, Portugal).

Experimental exposures

The exposure and data collection timeline is shown in Fig. 1. Zebrafish embryos of different developmental stages: 256-cell (2.5 h post-fertilization-hpf), 50% epiboly (5.5 hpf), and 1–4 somites phases (10.5 hpf) (Kimmel et al. 1995), and with intact chorions were exposed to freshly prepared solutions of ketamine for 20 min at a density of 30 embryos per 50-mL beakers and incubated at 28 ± 0.5 °C. The ketamine concentrations selected for this study were previously described to correspond to physiological concentrations that induced sedation (0.2 mg mL^{-1}), loss of equilibrium (0.4 mg mL^{-1}) and loss of response to a painful stimulus (0.8 mg mL^{-1}) in adult zebrafish (based on a pilot study and previously published work (Zakhary et al. 2011)) and to induce teratogenic effects in zebrafish embryos (Felix et al. 2014). Embryo water was used as negative control. Following treatment exposures, embryos were triple-rinsed with embryo water and allowed to develop at 28 ± 0.5 °C until they reached 144 hpf. During this period, dead individuals and debris were removed and water was replaced daily to maintain water quality. At least five independent replicates were performed for each developmental phase exposure.

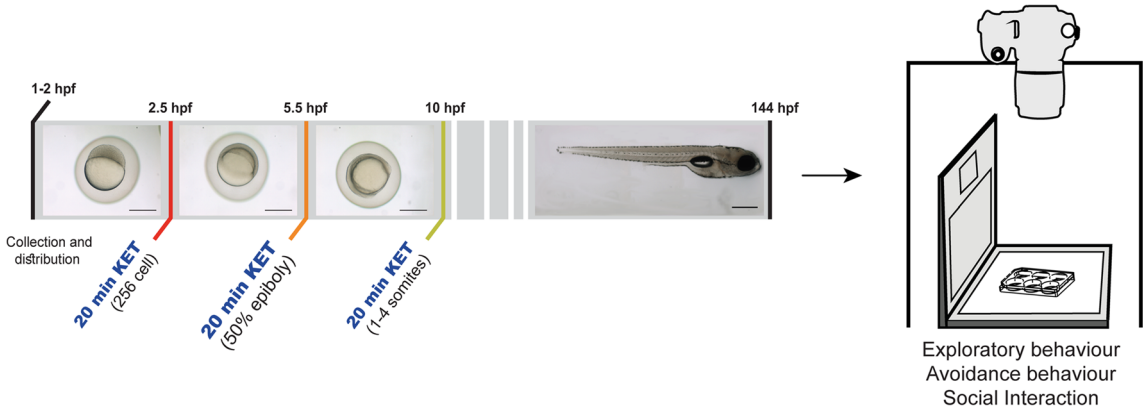

Fig. 1 Schematic diagram showing the experimental protocol of the study. Thirty embryos were independently exposed to ketamine concentrations (0.2, 0.4, or 0.8 mg mL^{-1}) or embryo water (control group) during 256 cell (2.5 hpf), 50% epiboly (5.5 hpf), or 1–4 somites (10.5 hpf) stage for a period of 20 min. At 144 hpf, larvae were allocated to six-well plates in order to evaluate exploratory, avoidance, and social behaviors. *Scale bar* represents 500 μm

Behavioral testing

All behavioral trials were carried out during the light period in an area at room temperature (25 °C). Before testing, the early developing larvae (144 hpf) were examined for malformations under an SMZ 445 stereomicroscope (Nikon, Japan) and all larvae exhibiting visible malformations were excluded from the behavioral testing assays (Felix et al. 2014). Behavioral testing consisted of recording larvae in 6-well plates. Each well of a 6-well F-bottom multiwell plate (Greiner Bio-one item-No. 657102, 35 mm diameter) was filled with 5 mL of melted 0.5% agarose to improve the optics at the edge of each well (Creton 2009). Once solidified, a circular portion was stamped out using a sharp stainless steel ring (27 mm diameter, 5 mm deep, and 1.5 mm thick) to create a circular swimming area and to avoid shadows and blind spots in the swimming area. In order to minimize differences in experimental timing during the testing period, experiments were performed in such a way that all groups were equally present in each well plate. Five independent replicates of 5 (for social behavior) or −6 larvae were used for each developmental phase exposure and ketamine concentration in the following behavioral assessments.

Video acquisition

The acquisition system used was previously described and implemented for zebrafish behavioral testing (Creton 2009; Pelkowski et al. 2011). The 6-well plate, without lid, containing zebrafish larvae was placed above an inverted 15.6″ laptop LCD screen (1366 × 768 pixel resolution, an average brightness of 173.6 cd m^{-2} and a contrast of 208:1 with a black level of 0.83 cd m^{-2}). A translucent cover (Leitz ColorClip 41740089) was used as an LCD diffuser film to avoid moiré patterns. Videos were captured from about 50 cm above at 30 frames per second (fps) with a Sony Nex-5 digital camera (14.2 megapixel APS-C CMOS sensor, Sony International, Europe) with a zoom lens (Sony SEL1855, E 18–55 mm, F3.5–5.6 OSS zoom). For video acquisition, a single 6-well plate was recorded at a maximum resolution of 1920 × 1080.

Locomotor activity and thigmotactic behavior

The spontaneous swimming behavior of zebrafish larvae following embryonic exposure to ketamine was evaluated at 144 hpf. Each well of the 6-well plate was filled with 3 mL of embryo water and a single larva was carefully transferred and released in each well center. Following 5 min of acclimation, the locomotor performance was recorded during a 10-min session and the following behavior patterns were measured: mean speed, total distance moved, mean distance to the center zone (i.e., to a 5-mm radius circle drawn in the center of the well) to assess thigmotaxis related to anxiety-like behaviors, percentage of time active and mean absolute turn angle. The mean absolute turn angle was computed as a mean of the differences between turn angles from a previous to the next frame across all the frames in the interval, and its value is always positive/absolute. Changes in turn angle may demonstrate a disorganized pattern of swimming, which could be a response to stress, or an indication of different morphological development (Danos and Lauder 2007).

Avoidance behavior

Larvae were analyzed regarding their ability to exhibit avoidance response to a visual stimulus using a similar approach to the one previously described and validated for zebrafish larvae (Pelkowski et al. 2011). Briefly, one 144 hpf larva was released into the center of each well and, after 5 min of acclimation, they were tested by alternating a 5-min period of a white background with a 5-min period of a red bouncing ball. This procedure was repeated two times. The bouncing ball (1.35 cm diameter) is recognized as an aversive stimulus (Richendrfer and Creton 2013) and was created in Microsoft PowerPoint using an

animated presentation. The ball was shown at the bottom half of the well and moving left-right-left at a speed of 1 cm s^{-1} over a straight 2 cm trajectory; furthermore the percentage of time spent in each zone (down vs up) was analyzed. During the periods when the bouncing ball was presented, a stationary ball was also placed in the upper half to counter-balance for brightness and color of the red bouncing ball, and to ensure that the larva was only reacting to the movement. To facilitate post-production, the Red-Green-Blue (RGB) values for the red balls were set at 255, 0, 0 and the white background was set at 255, 255, and 255. The post-production processing was addressed in Adobe After Effects CS5 (Adobe Systems, San Jose, USA).

Social behavior analysis

The social interaction between larvae was evaluated at 144 hpf according to established protocols (Richendrfer et al. 2012). Briefly, 5 larvae from the same exposure batch were transferred to the center of each well of a 6 well plate. After a 5 min of habituation period, the behavior of experimental groups was recorded for 10 min. The average inter-individual distance (IID) and the nearest neighbor distance (NND) were computed based on the x-y coordinates associated with the behavior quantification software according to formulas and descriptions described elsewhere (Miller and Gerlai 2012).

Video tracking and data processing

The quantification of zebrafish larvae behavioral parameters was achieved using TheRealFishTracker, a software application built for the Gerlai lab at the University of Toronto Mississauga by James McCrae (Buske and Gerlai 2014). This has been successfully employed to study embryonic zebrafish behavior (Buske and Gerlai 2011a, b). The software samples each video at a rate of 29 fps and records real-time precise location data (x-y coordinates) for each fish by comparing the present frame with the previous one, and allows the tracking of multiple subjects within the same setting. The software delivers files in plain text format which were afterward analyzed within the software or in a third-party software, such as Microsoft Excel, allowing the computation ofvarious behavioral processes. The confidence threshold was set at 30 and the mean filter size at 3 pixels; values were achieved by trial and error until the software accurately detected and followed larvae movement.

Statistical analyses

Based on a previous study in zebrafish larvae exposed to a teratogen where similar behavioral approaches were used (Clift et al. 2015), it was assumed that a reduction of 15% in swim speed would be pharmacologically significant. A sample size calculation was performed with the G*Power 3 (University of Düsseldorf, Germany) and it was determined that a total sample size of 12 (3 replicates per group) would be necessary for such difference to be detected, with an alpha error of 0.05 and a power of 95%. In order to increase the statistical power and accuracy of the analysis, biological replicates were increased. Before hypothesis testing, the normal distribution and homogeneity of the data were confirmed by Kolmogorov-Smirnov and Levene's tests, respectively. Data from the different groups were compared by a non-parametric independent samples Kruskal–Wallis test for non-normal distribution variables followed by Dunn's pairwise comparison tests and data expressed as median and interquartile range or by one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison tests for variables with normal distribution and data expressed as mean ± standard deviation. The dependent sample Student's *t* test was used to compare the statistically significant differences between an animal behavior when an aversive stimulus is presented or not. In all cases, statistical analyses were carried out using SPSS for Windows (Version 22.0; Chicago, IL, USA) and differences were considered significant at $p < 0.05$.

Results

Effects of ketamine exposure on exploratory behavior

As shown in Fig. 2, the spontaneous swimming behavior was only significantly altered in the distance to the center. Ketamine exposure at 50% epiboly induced an increment of the distance to the center from 0.54 (0.39–0.68) cm in the control group to 0.95 (0.84–1.07) cm for 0.2 mg mL^{-1} ($p = 0.002$), 1.04 (0.82–1.09) cm for 0.4 mg mL^{-1} ($p = 0.001$) and to 0.83 (0.73–0.94) cm for 0.8 mg mL^{-1} ($p = 0.028$). Similarly, 1–4 somites-exposed larvae were also affected, presenting increased distance values from control to the center: 0.87 (0.82–1.06) cm for 0.2 mg mL^{-1} ($p = 0.007$), 0.85 (0.79–1.20) cm for 0.4 mg mL^{-1} ($p = 0.003$) and 0.94 (0.87–1.07) cm for 0.8 mg mL^{-1} ($p = 0.003$). Still, a non-significant dose-dependent decrease in the mean speed of 256-cell-exposed embryos was observed when compared with the control group measures ($p = 0.079$ for the 0.8 mg mL^{-1} group). The absolute turn angle increased in larvae exposed to the highest dose during 50% epiboly ($p = 0.051$) and 1–4 somites stages ($p = 0.064$) compared with the control animals. The ketamine-treated groups behaved similarly regarding any of the parameters measured.

Ketamine effects on avoidance behavior

The avoidance behavior to a visual stimulus following exposure during the 256-cell, 50% epiboly, and 1–4 somites stages was analyzed in larvae at 144 hpf (Fig. 3). When spontaneous swimming behavior was assessed, without the presence of the aversive stimulus, zebrafish larvae presented neither a preference for the upper nor for the bottom area of the well. When the bouncing

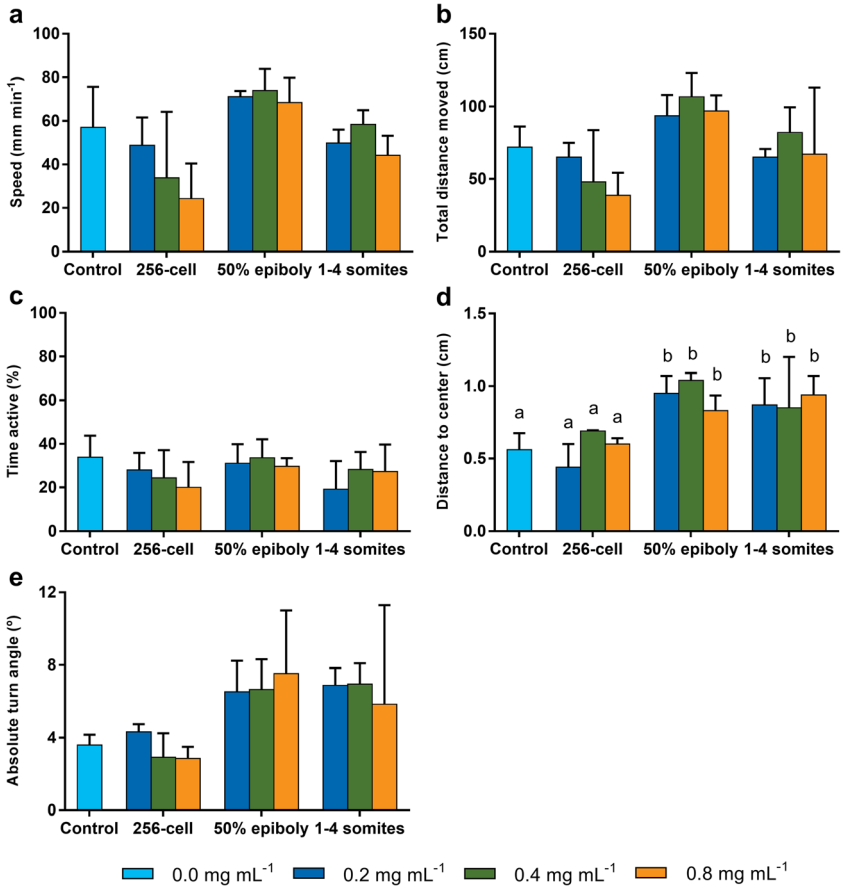

Fig. 2 Ketamine early exposure effects on exploratory behavior at 144 hpf larvae. **a** Swim speed. **b** Total distance moved. **c** Percentage of time larvae were active. **d** Distance to the center of the well. **e** Mean absolute turn angle. Values were expressed as mean ± SD (graph **c**) or median and interquartile range (graphs **a**, **b**, **d**, and **e**). Data from at least four independent replicate exposures (n = 4 replicates with six animals each per group). Different *lowercase letters* indicate significant differences between groups ($p < 0.05$, one-way ANOVA followed by Tukey's multiple-comparison test or Kruskal–Wallis test followed by Dunn's test) at each developmental stage

ball was presented in the bottom half of the well, there was a general increase in the time spent in the non-stimulus area (upper half) in all groups. A significant increase in the upper preference in the control (p = 0.023), and 256-cell exposed embryos (p = 0.015, p = 0.014, and p = 0.007 for 0.2, 0.4, and 0.8 mg mL^{-1}, respectively) was observed, suggesting an

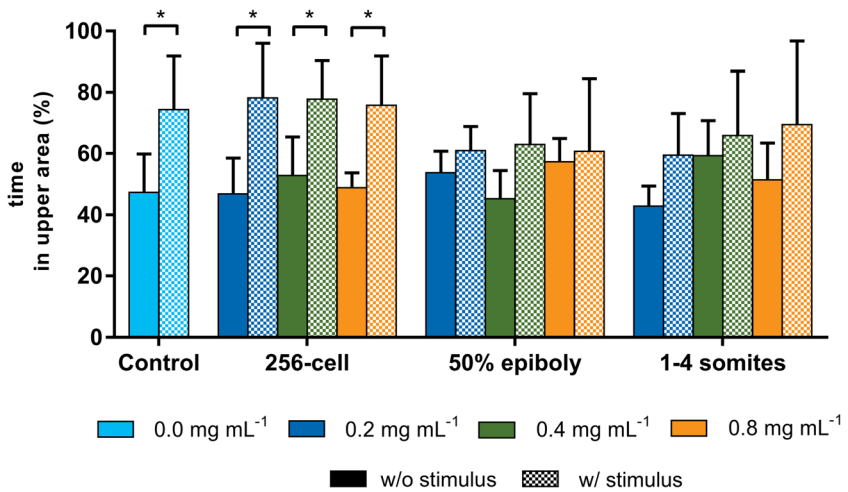

Fig. 3 Assessment of 144 hpf larvae avoidance behavior to a visual stimulus following early exposure to ketamine. Percentage (%) of time that each group from each stage spent in the upper area of the well without (*filled bars*) or with (*square pattern bars*) the presentation of an aversive stimulus (*bouncing ball*) in the bottom area of the well. The values are showed as mean ± SD from at least five independent replicate exposures (n = 5 replicates with six animals each per group). No significant differences were observed between groups ($p > 0.05$) without the *bouncing ball*. *Asterisks* indicate significant difference between groups before and after presentation of stimulus (dependent sample t test: *$p < 0.05$ and **$p < 0.01$)

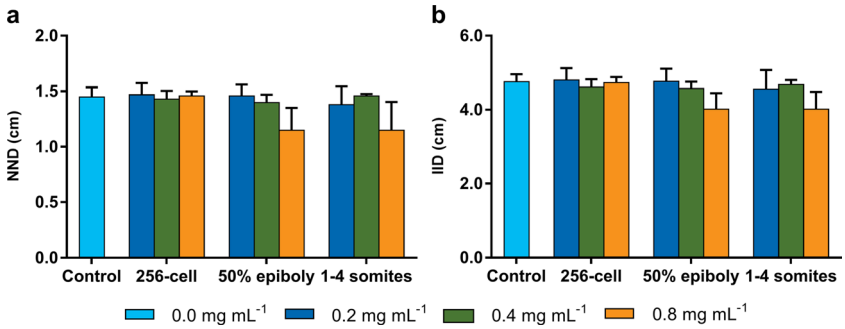

Fig. 4 Larvae social behaviors at 144 hpf after ketamine exposure at embryonic stages. **a** NND, nearest neighbor distance. **b** IID, inter-individual distance. The values are presented as median and interquartile range of five replicates of five larvae per group in each developmental exposure. No significant differences were observed between groups

avoidance response to the red ball stimulus. Larvae from 50% epiboly and 1–4 somites exposed-embryos had a non-significant increase in the time spent in the non-stimulus area.

Social behavior

Social behavior was evaluated quantifying the NND (nearest neighbor distance) and the IID (inter-individual distance) (Fig.4a, b). According to the results of the analyzed parameters, no interference was observed on the social cohesion of larvae following exposure to ketamine at early developmental phases.

Discussion

The present study was intended to explore possible behavioral alterations in zebrafish larvae related to abnormal brain processes following a short-exposure period to ketamine during the early stages of embryonic development (256-cell, 50% epiboly, and 1–4 somites). The rationale for this testing was related to the fact that ketamine had a potential teratogenic effect in 256-cell-exposed embryos, with effects still detected in 144 hpf larvae (Felix et al. 2014). Moreover, a relationship between later behavioral abnormalities and early developmental effects following chemical exposure has been proposed (Reif et al. 2016). The results of the experiments showed that a short-period of ketamine exposure during the 256-cell stage induced a slight decrease in the mean speed, while exposure during the 50% epiboly and 1–4 somites stages induced an increase in the distance to the center of the arena, suggesting the induction of anxiety-like behaviors and a decrease in avoidance behavior. The parameters of social behavior were not altered.

Ketamine has been described to alter gill movement, stress responses, and circling behavior in adult zebrafish (Zakhary et al. 2011) and larvae (Suen et al. 2013), as well asto induce altered startle reflex in zebrafish larvae (Burgess and Granato 2007; Wolman et al. 2011). Long-term behavioral alterations and its implications after the early developmental exposure of embryos to ketamine are still to be clarified. The assembly of the central nervous system (CNS) in the early stages of zebrafish is a complex biological process that relies on a functional cooperation between gene regulation, signaling pathways and electrical activity-driven mechanisms (Root et al. 2008). Also, its development occurs as a function of time from the blastula to more developed stages and relies on complex signaling pathways active at each developmental phase (Patthey and Gunhaga 2014; Schmidt et al. 2013). Disturbance of early cell fate and cellular rearrangements affects the major morphogenetic processes, resulting in malformed phenotypes (Solnica-Krezel et al. 1996), as pattern alterations of embryonic and early larval locomotion (Granato et al. 1996).

In this study, a dose-related tendency to decrease the mean speed was observed in the larvae exposed to ketamine during the embryo stage of 256-cell. It was previously shown that exposure of ketamine during this stage induces cumulative tail and spine skeletal deformities at 144 hpf (Felix et al. 2014) which may be the reason for the current observations. Ketamine also affects the differentiation of motor neurons (Kanungo et al. 2013), reducing the spine motor and sensory neurons in zebrafish (Cuevas et al. 2013). However, these studies exposed the animals to ketamine at later stages (28 hpf), when sensory-motor reflexive circuits are becoming functional (Kimmel et al. 1995). In the present study, the lack of statistical significance regarding locomotor parameters may be related to the exclusion of larvae with visible deformations before behavioral assay. Thus, further research is needed to understand the nature of the observed effects in the ketamine-exposed 256-cell embryos.

Nonetheless, the most significant findings of the current study were observed when zebrafish embryos were exposed to ketamine during the 50% epiboly and 1–4 somites stages. A significant increase in the distance to the center of the well was perceived in all ketamine exposures as well as a lack of preference for the area where a visual stimulus is not presented, indicating a lack of avoidance response. Thus, early exposure to ketamine affected anxiety-like and fear-like behaviors in 144 hpf larvae. Thigmotaxis has been used as an anxiety index in early zebrafish larvae, which usually has a preference for the edge of a circular well (Colwill and Creton 2011; Richendrfer et al. 2012; Schnorr et al. 2012). Regarding the presence of an aversive stimulus, zebrafish react with an adaptive escape reaction (Kalueff et al.

2013). Based on these, ketamine exposure at these stages may be related to the disruption of the normal development of the cerebral zones responsible for controlling emotional behaviors. Although zebrafish were exposed at very early stages, ketamine may influence neuronal induction, as it takes place around the onset of gastrulation by the interaction between various signaling pathways that regulate developmental processes such as proliferation, differentiation, migration and cell death (Aizawa 2013), supporting our results at the 50% epiboly and 1–4 somites stages. Without disregarding other mechanism and areas, it is hypothesized that ketamine may also interfere with the development of the habenula area. This has been described as a regulator of zebrafish fear/anxiety reactions (Lee et al. 2010; Mathuru and Jesuthasan 2013). Indeed, when neural firing is inhibited or when synaptic efficacy is reduced in the habenula, 144 hpf zebrafish show deficits to respond to a stressful stimulus (Lee et al. 2010). Moreover, in rats, chemical-induced lesions in the lateral habenula induced anxiogenic- and a panicolytic-like behaviors (Pobbe and Zangrossi 2008), similar to our observations. Furthermore, Sonic hedgehog (Shh) signaling pathway has been shown to play a crucial role in regulating the specification of the habenula (Chatterjee et al. 2014), being continuously required, at least until 24 hpf, for the production of habenular neurons (Halluin et al. 2016). In our previous study (Felix et al. 2016), exposure to ketamine during 50% epiboly induced an increase in Shh expression at 8 and 24 hpf (tendency) and at 144 hpf, while exposure at the 1–4 somites stage induced a dose-dependent increase in Shh expression by 24 hpf. Taking into account the important role that this signaling pathway has in the development of the diencephalon, we believe that ketamine exposure at these stages disrupted diencephalic development through changes in this signaling pathway; therefore, contributing to an aberrant habenular network function and consequently to the results obtained in this study. Still, knowledge on the involvement of signaling pathways and the effect of drugs during the establishment of the central nervous system is limited and further investigation will be necessary to address specifically how the different signaling pathways act during habenular development following exposure to a teratogenic agent, such as ketamine. The fact that ketamine induced an increase on anxiety-like behavior, but a reduced avoidance response, supports the idea that anxiety and fear are behavioral states with different processes associated (Richendrfer et al. 2012).

There were no differences between the control and ketamine-treated groups regarding the distance between the nearest neighbor and between the different individuals. In adults, ketamine induced a larger inter-fish distance, indicating lower anxiety or impaired social interaction compared with non-treated animals (Riehl et al. 2011). However, Mahabir and Buske described that social cohesion increases with age, and that the AB strain larvae have no shoaling at 7 dpf (Buske and Gerlai 2011b; Mahabir et al. 2013), supporting the lack of differences in this study.

It is noteworthy that in this study, zebrafish embryos were exposed to ketamine in developmental phases where there is no expression of endogenous glutamate receptors, which are only detected from 24 hpf onwards (Cox et al. 2005; Hwang et al. 2009; Klee et al. 2012). Thus, ketamine may not be limited to NMDA antagonism as this anesthetic also interacts with other neurotransmitter systems (Kohrs and Durieux 1998). In fact, catecholamine systems can be detected in zebrafish as early as 1 hpf, from maternal sources, and after 4 hpf following zygotic activation (Steele et al. 2011; Tufi et al. 2016). Moreover, other neurotransmitter systems such as the opioid system can be detected as early as 3 hpf (Sanchez-Simon and Rodriguez 2008). The early expression of opioid receptors seems to be required for neuronal differentiation (Kim et al. 2006; Sanchez-Simon and Rodriguez 2008) and the functional pharmacology of zebrafish opioid receptor system is similar to those of mammalian receptors (Marron Fdez de Velasco et al. 2009). Several lines of evidence in rodent models support the involvement of the opioid receptors in the regulation of emotion-related behaviors (Perrine et al. 2006) and in the development of the CNS (Narita et al. 2006). Thus, ketamine exposure shortly after the early expression of these receptors (in 50% epiboly and 1–4 somites) can also be a possible factor affecting early neurogenesis contributing to later behavioral deficits, as observed. In accordance, motor anomalies and defective thigmotaxis associated with defects in the differentiation of neural progenitors have already been reported in zebrafish (Pietri et al. 2013).

Nevertheless, ketamine may also be interfering with other cellular mechanisms. For instance, early developmental stages are known to be controlled by intracellular calcium signals (Webb and Miller 2000) and ketamine can block calcium oscillations during neuronal developmental processes, at concentrations superior to 3 mM (proximally 0.7 mg mL^{-1}) (Huang et al. 2013). During the earlier developmental stages of neurogenesis, calcium signaling pathways play crucial roles being involved in the neural induction and differentiation (Leclerc et al. 2012; Toth et al. 2016; Webb and Miller 2000). In zebrafish, these signaling pathways start at the end of the blastula stage and have their peak during gastrulation (Leclerc et al. 2012; Toth et al. 2016; Webb and Miller 2000). In addition to the imbalance in calcium signals to be involved in long-term development consequences (Creton et al. 1998; Ozil et al. 2006), changes in neuronal fate following calcium oscillations have been reported (Ben-Ari and Spitzer 2010). Moreover, a relationship exists between calcium and the activation of other signaling pathways that regulate neuronal gene expression (Wilder et al. 2009). Furthermore, the expression of a large number of genes and proteins critical for proliferation, migration, and differentiation of neural cells are also calcium-dependent (Toth et al. 2016). Therefore, an inefficient neural gene regulation after drug exposure may result in impairment or delay of the locomotor system development (Padilla et al. 2011).

Altogether, the results of the current study provide, to the best of our knowledge, the first evidence of stage-dependent long-term behavioral changes, namely anxiety- and fear-like behaviors, following exposure to an anesthetic at early developmental stages. The results of this study suggest that the exposure of zebrafish embryos to different concentrations of ketamine (sub-to anesthetic concentrations) at the period of 50% epiboly and 1–4 somites may have behavioral implications later in 144 hpf larvae. Although there are differences between species, these results enhance the need for more studies to understand the molecular mechanisms which may highlight the clinical implications of neurodevelopmental risks of early-life exposure to ketamine.

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