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Acetyl L-carnitine protects motor neurons and Rohon-Beard sensory neurons against ketamine-induced neurotoxicity in zebrafish embryos

Elvis Cuevas^a, William J. Trickler^{a,b}, Xiaoqing Guo^a, Syed F. Ali^a, Merle G. Paule^a, and Jyotshna Kanungo^{a,*}

^aDivision of Neurotoxicology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

^bToxicologic Pathology Associates, 3900 NCTR Road, Jefferson, AR 72079, USA

Abstract

Ketamine, a non-competitive antagonist of N-methyl-D-aspartate (NMDA) type glutamate receptors is commonly used as a pediatric anesthetic. Multiple studies have shown ketamine to be neurotoxic, particularly when administered during the brain growth spurt. Previously, we have shown that ketamine is detrimental to motor neuron development in the zebrafish embryos. Here, using both wild type (WT) and transgenic (*hb9:GFP*) zebrafish embryos, we demonstrate that ketamine is neurotoxic to both motor and sensory neurons. Drug absorption studies showed that in the WT embryos, ketamine accumulation was approximately 0.4% of the original dose added to the exposure medium. The transgenic embryos express green fluorescent protein (GFP) localized in the motor neurons making them ideal for evaluating motor neuron development and toxicities in vivo. The *hb9:GFP* zebrafish embryos (28 h post fertilization) treated with 2 mM ketamine for 20 h demonstrated significant reductions in spinal motor neuron numbers, while co-treatment with acetyl L-carnitine proved to be neuroprotective. In whole mount immunohistochemical studies using WT embryos, a similar effect was observed for the primary sensory neurons. In the ketamine-treated WT embryos, the number of primary sensory Rohon-Beard (RB) neurons was significantly reduced compared to that in controls. However, acetyl L-carnitine co-treatment prevented ketamine-induced adverse effects on the RB neurons. These results suggest that acetyl L-carnitine protects both motor and sensory neurons from ketamine-induced neurotoxicity.

Keywords

Ketamine; Motor neuron; Rohon-Beard neuron; Acetyl L-carnitine; Transgenic zebrafish

*Corresponding author at: Division of Neurotoxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA. Tel.: +1 870 543 7591; fax: +1 870 5437143. jyotshnabala.kanungo@fda.hhs.gov (J. Kanungo).

Conflict of interest

None.

1. Introduction

Ketamine [2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone] was first characterized in 1965 as having anesthetic properties (Craven, 2007). Recent findings indicate that anesthetics that act as NMDA receptors induce widespread neuronal apoptosis in the immature mammalian brain (Ikonomidou et al., 1999; Liu et al., 2013; Wang et al., 2013; Young et al., 2005; Zhang et al., 2008). Reports also show that exposure of the developing brain to a clinically relevant cocktail of anesthetics that has both NMDA antagonist and GABA mimetic properties results in an extensive pattern of neuroapoptosis, and subsequent cognitive deficits (Olney et al., 2002b). Several reports have illustrated that ketamine can induce neuronal apoptosis when administered in high doses and/or for prolonged durations during susceptible periods of development in rodents (Maxwell et al., 2006; Olney et al., 2002a; Wang et al., 2005) and primates (Haberny et al., 2002; Slikker et al., 2007a; Wang et al., 2006) and these effects can manifest on later disruptions in cognitive function (Paule et al., 2011). To minimize risks to children exposed to anesthesia, it is paramount to understand how anesthetic drugs affect the developing nervous system and whether those effects can be ameliorated or prevented.

Acetyl L-carnitine is a member of the family of carnitines, a group of natural compounds that is essential for β -oxidation of fatty acids in mitochondria to generate ATP (Bieber, 1988). It has been reported that acetyl-L-carnitine effectively prevents mitochondrial injury resulting from oxidative damage in vivo (Chang et al., 2002). Due to their intrinsic interaction with the bioenergetic processes, carnitines play important roles in mitochondrial-related functions and it has been suggested that carnitines may have neuroprotective actions in conditions of mitochondrial dysfunction and oxidative stress and possibly in neurodegenerative disorders, such as Parkinson's disease (Beal, 2004). Another important property of the carnitines is their ability to neutralize toxic acyl-CoA production in the mitochondria (Stumpf et al., 1985), which correlates with numerous diseases of the CNS including neurodegenerative diseases (Makar et al., 1995; Orth and Schapira, 2002; Rubio et al., 1998). Acetyl L-carnitine is also reportedly effective in reducing age-dependent mitochondria functional decay and in restoring mitochondrial membrane potential, cardiolipin content, metabolic oxygen (O_2) consumption, and β -oxidation of fatty acids (Gadaleta et al., 1998; Hagen et al., 1998, 2002a). For neurons at early developmental stages, co-administration of L-carnitine significantly diminished ROS (reactive oxygen species) generation and provided near complete protection of neurons from ketamine-induced neurodegeneration (Liu et al., 2013). Moreover, acetyl L-carnitine has been shown to protect neurons from inhalation anesthetic-induced neurotoxicity in rat cortical neurons (Zou et al., 2008).

The zebrafish maintain the typical complexity of vertebrate systems and accumulating evidence advocates its use in several areas of research with the prospect of extrapolating findings to other vertebrates and humans (Briggs, 2002; Parng et al., 2002; Powers, 1989; Vascotto et al., 1997). This is particularly intriguing given their small size, prolific reproductive capacity and ease of maintenance thus making it an ideal model for safety assessment of drugs (Ali et al., 2011; de Esch et al., 2012; Knudsen et al., 2011). Moreover, detailed automatic and manual annotation provides evidence of more than 26,000 protein-

coding genes, the largest gene set of any vertebrate so far sequenced, while comparison to the human reference genome shows that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). There are 10 NMDA receptor subunits found in zebrafish (Cox et al., 2005). There are two NMDAR1 genes (NR1.1 and NR1.2), and eight NMDAR2 genes, designated NR2A.1 and NR2A.2, NR2B.1 and NR2B.2, NR2C.1 and NR2C.2, and NR2D.1 and NR2D.2. Both the NR1 genes are expressed embryonically (Cox et al., 2005). NR1.1 is found in brain, retina and spinal cord at 24 h post fertilization (hpf). NR1.2 is expressed in the brain at 48 hpf but not in the spinal cord. Both paralogs of the NR2A are expressed at 48 hpf in the retina; only one paralog of the NR2B is expressed at low levels in the heart at 48 hpf (Cox et al., 2005). No NR2C paralogs are expressed embryonically while NR2D.1 is expressed in the forebrain, retina, and spinal cord at 24 hpf, and NR2D.2 is only found in the retina (Cox et al., 2005). Studies based on immunohistochemistry using available antibodies have shown that NR2A subunits are expressed in primary motor neurons and axons in zebrafish larvae (Todd et al., 2004). Expression of other subunits in both motor neurons and Rohon-Beard (RB) sensory neurons remains to be determined.

In zebrafish, the primary motor neurons arise from the ventral spinal cord during the first 24 h of development whereas secondary motor neurons arise later (Myers et al., 1986; Pike et al., 1992). During vertebrate nervous system development, apoptosis eliminates unnecessary cell types (Oppenheim, 1991). One such cell population is RB spinal sensory neurons. These are the early born primary sensory neurons of the central nervous system. The reason for the death of RB neurons during development is unknown. In amphibians, RB neurons die gradually and their death is coincident with the development of dorsal root ganglion (DRG) (Hughes, 1957). Zebrafish RB neurons are the equivalent of the amphibian RB neurons, having large cell bodies with huge nuclei and granular cytoplasm (Metcalf et al., 1990). Although zebrafish RB neurons begin showing signs of programmed cell death, such as DNA fragmentation, as early as the first day of development, the cells remain present for considerably longer and degenerate over a protracted period of time and unlike in amphibians, independent of any developmental link to DRG neurons (Williams et al., 2000).

Previously, we reported on the developmental toxicity of ketamine on motor neurons in zebrafish embryos (Kanungo et al., 2013). Importantly, in another study we also showed that acetyl L-carnitine counteracts ketamine's negative effects on heart rate and ERK (MAPK) activity in zebrafish embryos (Kanungo et al., 2012). In order to quantitate the effect of ketamine on motor neurons in vivo, hb9:green fluorescent protein (*hb9:GFP*) transgenic zebrafish embryos were used, in which the promoter of the transcription factor hb9 that is found in developing motor neurons of both mammals (William et al., 2003) and zebrafish (Cheesman et al., 2004; Park et al., 2004), drives GFP expression specifically in motor neurons (Flanagan-Steet et al., 2005; Nakano et al., 2005).

Here, we explored the effects of ketamine on both the motor neurons and primary sensory RB neurons. Additionally, we examined whether acetyl L-carnitine altered ketamine's effects on these neurons.

2. Materials and methods

2.1. Animals

Adult wild type (WT) and transgenic (*hb9:GFP*) zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center at the University of Oregon (Eugene, OR, USA). The fish were kept in fish tanks (Aquatic Habitats) at the NCTR/FDA zebrafish facility containing buffered water (pH 7.5) at 28 °C, and were fed daily live brine shrimp and Zeigler dried flake food (Zeiglers, Gardeners, PA, USA). Each 3 l tank housed 8 adult males or 8 females. Handling and maintenance of zebrafish were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the NCTR/FDA IACUC. The day–night cycle was maintained at 14:10 h, and spawning and fertilization were stimulated by the onset of light at 8:30 AM. For in-system breeding, crosses of males and females were set up the previous day with partitions that were taken off the following morning at the time of light onset at 8:30 AM. Fertilized zebrafish embryos were collected from the bottom of the tank as soon as they were laid. The eggs/embryos were placed in Petri dishes and washed thoroughly with buffered egg water (reverse osmosis water containing 60 mg sea salt (Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA) per liter of water (pH 7.5) and then allowed to develop in an incubator at 28 °C for further experiments.

2.2. Treatment of zebrafish embryos with ketamine and acetyl L-carnitine

Embryos (28–48 hpf) were exposed to ketamine and ketamine plus acetyl L-carnitine. For each experiment, three sets of 28 hpf dechorionated (manual dechoriation using a pair of watchmakers' forceps) embryos were used. Each set included 10 embryos placed in individual wells of six-well plates [$n = 30$ /each experimental group (pooled embryos from three wells)], each well containing 5 ml egg water and 10 embryos. Ketamine (ketamine hydrochloride from Sigma, St Louis, MO, USA; catalog no. K2753) was dissolved as a stock solution of 100 mg/ml in buffered egg water. The solution was made fresh and treatment (static exposure) at 0.5, 1.0 and 2.0 mM continued for 20 h. An untreated control group of 10 embryos/set ($n30$) was examined in parallel. Embryos were incubated at 28.5 °C. Based on our earlier studies (Kanungo et al., 2012), acetyl L-carnitine (Sigma, St Louis, MO) was used at 0.5 mM for co-treatment with ketamine.

2.3. Determination of ketamine levels in zebrafish embryos using reverse phase HPLC

The embryos were collected in microcentrifuge tubes (1.5 ml), centrifuged in a microcentrifuge ($1700 \times g$) and the aqueous layer was discarded and replaced with fresh deionized water (0.5 ml) three additional times. After the final centrifugation, the embryo samples were alkalized with 0.35 ml of 0.2 mol/l borate buffer and sonicated at 30% intensity for 15 s and then extracted with dichloromethane: ethyl acetate (80:20 v/v) by mixing ($75 \times g$, 10 min). The samples were then centrifuged ($1500 \times g$, 3 min), the organic layer was transferred to a glass test tube, and the samples were extracted again using dichloromethane:ethyl acetate (80:20 v/v). The combined organic layer was back-extracted with perchloric acid (2 N), and the organic layer was discarded. The acidic aqueous layer was evaporated to dryness at 45 °C. The dried residue was reconstituted in 0.1 ml of mobile

phase and 50 μ l was injected onto the HPLC column, and the amount of ketamine was determined by a method described below.

The amount of ketamine was determined using a rapid HPLC method on a Waters 2695 separations module coupled to a Waters 2996 PDA (Milford, MA, USA). Briefly, the isocratic HPLC separation of ketamine was achieved on a SB-C18 Zorbax column (150 $\times$ 4.6 mm, 5 μ m) (Agilent, Santa Clara, CA, USA) using a simple mobile phase consisting of acetonitrile:0.03 mol/l phosphate buffer (23:77% v/v) adjusted to pH 7.2 at a flow rate of 1.5 ml/min. The effluents were monitored at 210 nm and quantified using the area under the peak from standard solutions dissolved in deionized water (0.75–200 μ mol/l). The accumulated amount of ketamine per embryo was empirically derived by determining the fraction of the dose absorbed in the embryo from triplicate samples. The data are expressed as means (amount/embryo) $\pm$ SDs.

2.4. Live embryo morphological assessment

Morphological features and spontaneous swimming movements of the embryos were examined and recorded using an Olympus SZX 16 binocular microscope and a DP72 camera.

2.5. Acridine orange staining

To detect apoptosis in live embryos, staining with the vital dye, acridine orange (AO, acridinium chloride hemi-[zinc chloride]) was performed (Abrams et al., 1993). Embryos were placed in 5 μ g/ml of AO (Sigma, St. Louis, MO) in embryo water after exposure to drugs. Thirty minutes after staining in dark, embryos were washed with embryo water and viewed and imaged using the green filter with an Olympus SZX 16 microscope and a DP72 camera.

2.6. Live embryo imaging

Transgenic hb9–GFP zebrafish (Flanagan-Steet et al., 2005) used in this study are of the AB strain. The transcription factor hb9 is found in developing motor neurons of both mammals (William et al., 2003) and zebrafish (Cheesman et al., 2004; Park et al., 2004). In the *hb9–GFP* transgenic fish, motor neurons are labeled with strong neuron-specific expression of GFP under the control of the regulatory elements of the zebrafish hb9 gene (Flanagan-Steet et al., 2005; Nakano et al., 2005). Post-treatment with ketamine, images of the *hb9–GFP* transgenic embryos were acquired using an Olympus SZX 16 binocular microscope and a DP72 camera. Higher magnification images were acquired using a Nikon Eclipse 80i microscope and a Nikon DXM1200C digital camera. In embryos treated with ketamine for 20 h (static exposure), GFP-expressing spinal motor neurons in the trunk region (three hemisegments following the distal end of the yolk extension) were counted per specific hemisegments following a procedure used earlier (Kanungo et al., 2013, 2009). The GFP-positive neurons were visually counted in specific hemisegments when the particular region was finely focused under the microscope so that individual neurons were clearly visible. These numbers represent relative numbers of motor neurons in the embryos since it is not possible to visualize all the GFP-positive cells, especially when there could be overlapping

GFP signals. The values from 10 embryos per experimental group were averaged to obtain the number of neurons/hemisegment.

2.7. Whole-mount immunohistochemistry for Rohon-Beard sensory neurons

For whole-mount immunohistochemistry to identify the Rohon-Beard (RB) sensory neurons, wild type embryos, after drug treatment, were fixed in 4% paraformaldehyde overnight. Embryos were then washed extensively in phosphate-buffered saline (PBS) and stored in acetone at -20°C for 1 h. After a quick rinsing with water, embryos were extensively washed with PBS and blocked in 5% sheep serum and 2 mg/ml bovine serum albumin (BSA) in PBS for 2 h at room temperature. Embryos were exposed to the monoclonal Zn-12 antibody (Metcalf et al., 1990), a primary neuron marker in zebrafish, obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA). The antibody solution (1:100) also contained 0.05% Triton X-100 in PBS, and embryos were incubated overnight at 4°C with agitation. After exposure to the primary antibody, embryos were rinsed extensively with PBS and incubated with Texas Red-conjugated anti-mouse antibody (Jackson Labs, Bar Harbor, ME) at a dilution of 1:200 in PBS containing 5% sheep serum and 2 mg/ml bovine serum albumin at 4°C overnight in the dark. After several washings in PBS, embryos were examined and photographed using a Nikon Eclipse 80i microscope and a Nikon DXM1200C digital camera.

3. Results

3.1. Determination of ketamine levels in zebrafish embryos using reverse phase HPLC

Manually dechorionated 28 hpf WT embryos were exposed to various doses of ketamine (0.5, 1.0 and 2.0 mM) for 20 h. Internal ketamine concentrations in these embryos (exposed from 28–48 hpf), were determined using reverse-phase HPLC and were well within the limits of quantification after 20 h of static exposure (Fig. 1A). The mean amount of ketamine absorbed was determined to be 2.2 μM , 4.6 μM , and 8.4 μM per embryo, respectively. The percentage of the original ketamine dose absorbed per embryo did not vary significantly in the three ketamine-treated groups ranging from 0.42% to 0.46% (Fig. 1B). These results indicate that although the levels of ketamine in the exposure water are at the millimolar level, the levels of ketamine actually accumulating in the embryos are significantly less.

3.2. Live embryo morphological assessment

Based on these data that at a 2.0 mM dose, ketamine accumulation is only 0.4% of the treated dose, we used 2.0 mM ketamine for our next experiments. Although no drastic morphological abnormalities were noted after ketamine or ketamine plus acetyl L-carnitine treatment (Fig. 2), ketamine-treated embryos were completely anesthetized as determined by their immobility and lack of response to touch (Fig. S1). On the other hand, embryos co-treated with 2 mM ketamine and 0.5 mM acetyl L-carnitine were indistinguishable from the control; they were not anesthetized (Fig. S1). Control embryos as well as embryos treated with both ketamine and acetyl L-carnitine showed spontaneous swimming ability while ketamine-treated embryos remained immobile even when the 6-well plate was manually moved to invoke a response.

3.3. Assessment of the effect of ketamine on motor neurons in vivo

In order to assess the effect of ketamine on the motor neurons of the developing zebrafish nervous system in vivo, we used a transgenic zebrafish line (*hb9:GFP*) that has motor neurons specifically identified by the expression of GFP. Exposure to ketamine for 20 h (28–48 hpf) resulted in a reduction in the GFP-positive motor neuron population when compared to control treated embryos (Fig. 3). Since it is not possible to visualize or count individual cranial motor neurons, using fluorescence microscopy, quantification of GFP-positive spinal motor neurons in specific hemisegments in these embryos was accomplished. By visually counting the neurons in the three hemisegments distal to the yolk extension and obtaining the mean value, the average neuron numbers per hemisegment were calculated. Compared with controls (Fig. 3Aa), there was a significant reduction (30%) in the number of spinal motor neurons in ketamine-treated embryos (Fig. 3Ab). In embryos treated with ketamine plus acetyl L-carnitine, the number of spinal motor neurons was restored, almost to control levels (Fig. 3Ac). The group data (Fig. 3B) suggest that ketamine's adverse effect on the motor neurons is prevented by acetyl L-carnitine.

3.4. Assessment of the effect of ketamine on primary sensory Rohon-Beard (RB) neurons in situ

In order to determine the effect of ketamine on the primary sensory neuron development, we examined the RB neurons following drug exposure. RB neurons are the primary sensory neurons to develop along the neural axis and may be the first to respond to drugs or chemicals due to their superficial location beneath the skin. We used whole mount immunohistochemistry using a specific antibody (Zn-12) to identify the RB neurons. In the control embryos, the RB neurons appeared to be distributed normally along the body axis (Fig. 4Aa). However, in the ketamine-treated embryos, there was a significant reduction in the number of RB neurons (Fig. 4Ab). Assessment of RB neurons at 48 hpf provided data on the relative effects of ketamine on these short-lived neurons and the results clearly show an adverse effect of ketamine. In embryos treated with ketamine plus acetyl L-carnitine, the number of RB neurons was restored to a significantly higher level (Fig. 4Ac). The relative numbers of RB neurons in the various groups (Fig. 4B) suggest that as in the case of motor neurons, ketamine's adverse effect on the RB neurons is also prevented by co-treatment with acetyl L-carnitine.

3.5. Acridine orange staining of embryos to assess apoptosis in vivo upon ketamine exposure

We stained live embryos with acridine orange (Fig. 5) to see if the neurotoxicity observed in ketamine-treated embryos resulted from neuroapoptosis and acetyl L-carnitine prevented it. Apart from a non-specific autofluorescence in the yolk and yolk extension with few positive signals at around it in the ketamine-treated embryos, we observed almost no differential cell staining in wild-type embryos or the embryos in the experimental groups. These studies showed no neuroapoptosis upon ketamine treatment.

4. Discussion

Ketamine is a dissociative anesthetic that produces anesthesia, analgesia, suppression of fear and anxiety, and amnesia and its effects are mediated by antagonism of NMDA receptors (NMDARs) (Anis et al., 1983). Activation of NMDARs is essential for long-term potentiation and spatial learning and memory (Malenka and Bear, 2004), and NMDAR blockade results in impaired synaptic plasticity manifested as adverse effects on learning and memory (Sakimura et al., 1995; Shimizu et al., 2000). It has been shown that higher doses of ketamine can induce neuroapoptosis in rodents (Maxwell et al., 2006; Olney et al., 2002a; Wang et al., 2005) and primates (Haberny et al., 2002; Slikker et al., 2007b; Wang et al., 2006) during early development. In agreement, we have previously reported that ketamine induces motor neuron toxicity in zebrafish embryos (Kanungo et al., 2013).

The current study on the effects of ketamine on zebrafish embryos provides further evidence that, after 20 h of exposure, 2.0 mM ketamine significantly reduces primary sensory RB neuron and spinal motor neuron populations. These data are concordant with the results in multiple reports (cited above) on mammalian studies (neurotoxicity induced by ketamine in rodents and monkeys). Based on our continuing studies showing ketamine accumulation in 72 hpf embryos (48 hpf embryos exposed for 24 h to 2 mM ketamine) is much less ($\sim 7 \mu\text{M}$ /embryo) compared to the 2.0 mM treatment dose (Trickler et al., 2013), we tested whether a similar phenomenon occurred in 28 hpf embryos that were treated for 20 h with 2.0 mM ketamine. The rationale behind this experiment was based on the notion that being younger by 20 h than the 48 hpf embryos, the skin of the 28 hpf embryos will be less complex and more permeable to ketamine, thus facilitating enhanced diffusion. The dose and duration were selected on the basis of our previous report on ketamine-induced cardiotoxicity in zebrafish embryos (Kanungo et al., 2012). In our studies, although ketamine dose was high in the embryo water, the real exposure turned out to be significantly lower; at 2.0 mM ketamine dose, each embryo only accumulated $8.4 \mu\text{M}$. It has been shown that, in postnatal day 7 rat pups, repeated doses of 20 mg kg^{-1} ketamine resulted in a blood level of $14 \mu\text{g ml}^{-1}$ concentration and induced neuroapoptosis in the dorsolateral thalamus (Scallet et al., 2004). The blood level of $14 \mu\text{g ml}^{-1}$ is approximately 7-fold greater than anesthetic blood levels in humans (Mueller and Hunt, 1998). The doses we used, 0.5 mM (1.37 mg ml^{-1} water), 1.0 mM (2.74 mg ml^{-1} water) and 2.0 mM (5.48 mg ml^{-1} water) are in the range of the effective doses that induce behavioral changes in zebrafish larvae (Burgess and Granato, 2007). However, when 2.0 mM ketamine was used, the real exposure for ketamine in our study was only $8.4 \mu\text{M}$ per embryo, a dose that is comparable to the reported neurotoxic dose in mammals.

In the current study, ketamine treatment began with 28 hpf embryos, when only NR1.1 and NR 2D.1 are found in the brain and spinal cord. NR1.2 is expressed in the brain at 48 hpf but not in the spinal cord (Cox et al., 2005), a time point when ketamine exposure was terminated in the present study. These time points (28–48 hpf) were chosen since the goal was to monitor ketamine's effects in vivo and beyond 48 hpf, excess pigmentation hinders visualization of the GFP signals. Although development of zebrafish pigmentation can be inhibited by early treatment of embryos with phenylthiourea to facilitate visualization of

tissues and organs and, therefore, GFP signals, phenylthiourea is a known toxic compound and was not used in our experiments.

While ketamine reduced the motor neuron and RB neuron numbers, acridine orange staining to detect apoptosis in vivo did not show any positive cells. These results suggested that the reduced number of neurons in ketamine-treated embryos may have resulted from slowed development or differentiation. Lack of acridine orange-positive cells in these embryos also indicates that neurons may have degenerated by a process other than apoptosis, possibly, necrosis. Further studies are required to determine the exact mechanism of ketamine-induced neurotoxicity. Interestingly, ketamine has been shown to induce both apoptosis (at low doses in a time-dependent manner) and necrosis (in higher doses) in neuronal as well as non-neuronal cells (Braun et al., 2010). Whether the ketamine dose in our studies would be considered high, so necrosis may have resulted but not apoptosis remains to be tested. Also, ketamine-induced apoptosis could have occurred earlier during the exposure duration of 20 h, which needs to be examined.

As a continuation of our previous study (Kanungo et al., 2013), although the focus of the present study, once again was on motor neurons; but this time, the emphasis was on attempting to reverse the ketamine-induced phenotype. Motor neuron development is a more sensitive indicator of neurotoxicity than the morphological end points used in early zebrafish embryos (Sylvain et al., 2010, 2011). We took advantage of the transgenic (*hb9:GFP*) line that makes motor neurons visible in vivo. Ketamine at 2.0 mM induced motor neuron toxicity in vivo as expected, while co-treatment with acetyl L-carnitine significantly attenuated this effect. A similar preventive effect of acetyl L-carnitine on ketamine's effects was also observed for the RB primary sensory neurons. Ketamine-induced attenuation of heart rate in zebrafish embryos has been shown to be normalized in the presence of acetyl L-carnitine (Kanungo et al., 2012). Whether a single specific signaling pathway culminating in different tissue-specific effectors or completely distinct pathways regulating specific/common effectors are triggered by acetyl L-carnitine remains to be elucidated.

Although the mechanisms of ketamine-induced neurotoxicity are not fully understood, mitochondrial dysfunction, oxidative stress and alterations in calcium homeostasis may be involved. Ketamine-induced apoptosis by mitochondrial pathway has been shown in neuronal cells (Braun et al., 2010; Yon et al., 2005) as well as non-neuronal (lymphocytes and hepatic) cells (Braun et al., 2010; Lee et al., 2009). Acetyl L-carnitine appears to be involved in the restoration of mitochondrial function and/or improved use of energy from glycolysis in cultured neuroblastoma cells treated with the neurotoxicant, 1-methyl-4-phenyl tetrahydropyridine (Mazzio et al., 2003). It has also been reported that rotenone-induced mitochondrial function inhibition can cause cell death in vitro in cultured rat cortical neurons that were, in part, prevented by the co-incubation of the cells with 1 mM acetyl L-carnitine (Virmani et al., 1995). Carnitine has also been shown to effectively inhibit mitochondrial injury induced by oxidative stress and mitochondria-dependent apoptosis in various types of cells (Furuno et al., 2001; Hagen et al., 2002b; Therrien et al., 1997). Acetyl L-carnitine also protects rat cortical neurons from anesthetic-induced apoptosis (Wang et al., 2007; Zou et al., 2008). Additionally, acetyl L-carnitine attenuated ketamine-induced behavioral alterations and body weight decrements in preweaning rats (Boctor et al., 2008)

and was found to exert efficient preventive effects in a cellular model of Parkinson's disease (Zhang et al., 2010). Beneficial effects of acetyl L-carnitine on cognitive and mitochondrial dysfunction have been shown in aging rats (Hagen et al., 2002a; Liu et al., 2002), as well. In our earlier study, we speculated that acetyl L-carnitine's reversal of ketamine-induced decrease in heart rate and ERK/MAPK activity could be mediated by calcium-modulated signaling (Kanungo et al., 2012). Moreover, acetyl L-carnitine has been shown to protect hippocampal neurons from hypoxia-induced apoptosis by activating NGF receptors that resulted in ERK/MAPK activation (Barhwal et al., 2008). Further studies in this context are needed to elucidate exactly how acetyl L-carnitine counteracts ketamine's adverse effects on the RB and motor neurons.

Taken together, our in vivo (for motor neurons) and in situ (for RB neurons) data suggest that ketamine is toxic to both sensory and motor neuron development in zebrafish and acetyl L-carnitine protects these neurons from ketamine-induced adverse effects. These results are in concordance with mammalian data suggesting that in zebrafish, ketamine and acetyl L-carnitine trigger physiologic responses similar to those observed in higher vertebrates. Thus, risk assessment of drugs using the zebrafish embryo model appears to be complementary to studies performed in higher order species and, may in some cases, provide a useful alternative.

5. Disclaimer

This document has been reviewed in accordance with the United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the FDA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ntt.2013.07.005>.

Fig. 1.

Ketamine accumulation (internal ketamine concentrations) in zebrafish embryos after static exposure to ketamine. Embryos at 28 hpf were exposed for 20 h to 0.5, 1.0 and 2.0 mM ketamine. Post-exposure, embryos were washed extensively with quick changes of embryo water three times. Reverse-phase HPLC was employed to measure ketamine concentrations in the embryos ($n = 30/\text{group}$). Data from three separate estimations were averaged and are presented as mean $\pm$ SD (bar). (A) Ketamine dose (μg) accumulated per embryo treated with 0.5 (2.2 ± 0.0002), 1.0 (4.6 ± 0.0002) and 2.0 mM ketamine (8.4 ± 0.0001); (B) percentage of the original ketamine dose accumulated per embryo.

Fig. 2.

Morphological effect of ketamine on zebrafish embryos. Wild type embryos at 28 hpf were treated with ketamine. After 20 h of treatment (48 hpf actual age), images of the live embryos were acquired. In this experiment, MS-222 was not used (a routine procedure) to immobilize the untreated control embryos for photography in order to avoid any interference with the effect produced by ketamine. The experiment was repeated three times with $n = 10$ for each group in each experiment. Lateral views of the embryos are shown with dorsal side up. Embryos in different experimental groups are (A) control, (B) 2.0 mM ketamine-treated, and (C) 2.0 mM ketamine plus 0.5 mM acetyl L-carnitine.

Fig. 3.

Effect of ketamine on spinal motor neurons in vivo. Transgenic (*hb9:GFP*) fish embryos (28 hpf) were treated with 2.0 mM ketamine. After 20 h of static exposure (48 hpf actual age), images of the live embryos (lateral views with dorsal side up and anterior side to the left) were acquired for the assessment of GFP fluorescence. (A) GFP-expressing motor neurons in the spinal cord are shown in control (a) and ketamine-treated (b), and ketamine plus acetyl L-carnitine-treated (c) embryos. Spinal motor neurons could be visualized individually under the microscope. GFP-positive motor neurons in specific hemisegments were counted. ANOVA and Holm–Sidak pair-wise multiple comparison post-hocs (Sigma Stat 3.1 for analysis) were used to determine statistical significance with significance (*) set at $P < 0.05$

(B). In each of three experiments, spinal motor neurons were counted in 10 embryos each from the control and ketamine-treated and ketamine plus acetyl L-carnitine-treated groups. Data were averaged and are presented as mean $\pm$ SD (bar).

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Fig. 4.

Effect of ketamine on primary sensory Rohon-Beard (RB) neurons. Wild type zebrafish fish embryos (28 hpf) were treated with 2.0 mM ketamine or 2.0 mM ketamine plus 0.5 mM acetyl L-carnitine. After 20 h of static exposure (48 hpf actual age), embryos were fixed in 4% paraformaldehyde overnight, blocked in 5% sheep serum and 2 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and incubated overnight (4 °C) with a mouse monoclonal antibody, Zn-12 (1:100). Embryos were then incubated in Texas Red-conjugated anti-mouse antibody at 4 °C overnight before being visualized for Zn-12-positive RB neurons. (A) Zn-12-positive RB neurons are shown in control (a) and ketamine-treated (b), and ketamine plus acetyl L-carnitine-treated (c) embryos. ANOVA and Holm–Sidak pair-wise multiple comparison post-hocs (Sigma Stat 3.1 for analysis) were used to determine statistical significance with significance (*) set at $P < 0.05$ (B). In each of three experiments, RB neurons in a specific region were counted in 10 embryos each from the

control and ketamine-treated and ketamine plus acetyl L-carnitine-treated groups. Data were averaged and are presented as mean $\pm$ SD (bar).

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Fig. 5. Acridine orange staining of embryos to detect apoptosis. Wild type zebrafish embryos (28 hpf) were treated with 2.0 mM ketamine or 2.0 mM ketamine plus 0.5 mM acetyl L-carnitine. After 20 h of static exposure (28–48 hpf), embryos were stained with the vital dye, acridine orange in order to detect apoptotic cells that would be fluorescent. Control (A), ketamine-treated (B) and ketamine plus acetyl L-carnitine-treated (C) embryos are shown.