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Research paper

Opposing effects of ketamine and acetyl L-carnitine on the serotonergic system of zebrafish

Bonnie L. Robinson, Melanie Dumas, Merle G. Paule, Syed F. Ali, Jyotshna Kanungo*

Division of Neurotoxicology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA

HIGHLIGHTS

- Low doses of ketamine do not alter 5-HT and 5-HIAA levels in zebrafish embryos.
- Anesthetic dose of ketamine reduces 5-HT levels and abolishes its metabolism to 5-HIAA.
- ALCAR reverses the effects of ketamine on the 5-HT level and metabolism.
- Anesthetic dose of ketamine reduces serotonin-positive cells in the brain.
- Ketamine and ALCAR are being tested as anti-depressants, these results indicate their possible modes of action on the 5-HT system.

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ABSTRACT

Ketamine, a pediatric anesthetic, is a noncompetitive *N*-methyl-D-aspartic acid (NMDA) receptor antagonist. Studies show that ketamine is neurotoxic in developing mammals and zebrafish. In both mammals and zebrafish, acetyl L-carnitine (ALCAR) has been shown to be protective against ketamine toxicity. Ketamine is known to modulate the serotonergic system in mammals. Here, we measured the levels of serotonin (5-HT) and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA) in the embryos exposed to ketamine in the presence and absence of ALCAR. Ketamine, at lower doses, did not produce significant changes in the 5-HT or 5-HIAA levels in 3 dpf (day post-fertilization) embryos. However, 2 mM ketamine (internal embryo exposure levels comparable to human anesthetic plasma concentration) significantly reduced 5-HT level, and 5-HIAA was not detectable indicating that 5-HT metabolism was abolished. In the presence or absence of 2 mM ketamine, ALCAR by itself did not significantly alter 5-HT or 5-HIAA levels compared to the control. Ratios of metabolite/5-HT indicated that 2 mM ketamine inhibited 5-HT metabolism to 5-HIAA whereas lower doses (0.1–0.3 mM) of ketamine did not have any effect. ALCAR reversed the effects of 2 mM ketamine not only by restoring 5-HT and 5-HIAA levels but also 5-HT turnover rate to control levels. Whole mount immunohistochemical studies showed that 2 mM ketamine reduced the serotonergic area in the brain whereas ALCAR expanded it with increased axonal sprouting and branching. These results indicate that ketamine and ALCAR have opposing effects on the zebrafish serotonergic system.

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1. Introduction

Ketamine, a pediatric anesthetic, is a noncompetitive antagonist of the *N*-methyl-D-aspartic acid (NMDA) receptor [22]. Although a dissociative anesthetic [10], ketamine renders analgesic and amnesic effects and its potential anti-depressant properties are also being investigated [3,6,30,36]. A number of studies on rodents and non-human primates have shown that treatment with high doses of ketamine or exposure for long durations during susceptible periods of development can induce neuroapoptosis [14,16,39]. Recently, ketamine's role as an anti-depressant has also been documented [29,38].

Acetyl L-carnitine (ALCAR) belongs to the family of carnitines, a group of naturally occurring compounds that is essential for β-oxidation of fatty acids in mitochondria to generate ATP [7]. ALCAR effectively prevents mitochondrial injury resulting from oxidative damage [33]. It is also suggested that carnitines have neuroprotective effects on conditions caused by mitochondrial dysfunction and oxidative stress and possibly in neurodegenerative disorders, such as Parkinson's disease [5]. Moreover, carnitines can neutralize toxic

* Corresponding author at: Division of Neurotoxicology, National Center for Toxicological Research, US Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA. Fax: +1 870 543 7143.
E-mail address: jyotshnabala.kanungo@fda.hhs.gov (J. Kanungo).

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acyl–CoA production in the mitochondria [41] that occurs in various CNS disorders, such as Parkinson's disease [32]. ALCAR is also effective in reducing age-dependent progressive mitochondrial dysfunction and can restore mitochondrial membrane potential, metabolic oxygen consumption and β-oxidation of fatty acids [15]. For neurons at early developmental stages, co-administration of ALCAR significantly diminished reactive oxygen species (ROS) generation and provided significant protection of neurons from ketamine-induced neurodegeneration [26]. Additionally, ALCAR has been shown to protect neurons from inhalation anesthetic-induced neurotoxicity in rat cortical neurons [52]. In zebrafish embryos, ALCAR offers protection against ketamine-induced cardiotoxicity and neurotoxicity [11,19].

Serotonin (5-hydroxytryptamine; or 5-HT) and monoamine oxidase (MAO) are involved in a number of physiological functions and pathological conditions. Zebrafish serotonergic system and its development are similar to those of higher order vertebrates [37]. Therefore, zebrafish embryos/larvae can be a good model system to study the 5-HT system. For its small size, prolific reproductive capacity and easy maintenance, zebrafish possess the typical complexity of vertebrate systems, and therefore, have been used in several areas of research with the prospect of extrapolating findings to mammals including humans [8,20,24,34].

In rats, ketamine inhibits the uptake of serotonin (5-HT) both in vitro [4] and in vivo [27]. In the rat brain, ketamine inhibits 5-HT synthesis and metabolism in vivo [28]. Ketamine exerts its antidepressant effects only at lower doses while higher doses induce anesthesia [29]. Zebrafish embryos have a functional 5-HT system with MAO mRNA expression in the brain beginning at 24 h post-fertilization (hpf) and enzyme activity at 42 hpf [37]. In mammalian models and patients, the role of 5-HT in depressive disorders and the modulation of the 5-HT system by ketamine have been reported [46,49,50]. In the present study, we examined whether ketamine and ALCAR evoke a response in the 5-HT system in the zebrafish embryos in terms of 5-HT biosynthesis and metabolism.

2. Materials and methods

2.1. Animals

Adult wild type (WT) zebrafish (*Danio rerio*, AB strain) were obtained from the Zebrafish International Resource Center (www.zirc.org) (Eugene, OR, USA). The fish were kept in fish tanks (Aquatic Habitats, FL, USA) 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. 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 7:30 AM to stimulate spawning and fertilization. Fertilized zebrafish eggs were collected from the bottom of the tank as soon as they were laid. The eggs 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 28.5 °C for later use.

2.2. Reagents

Ketamine hydrochloride was purchased from Vedco, Inc. (St. Joseph, MO, USA). Acetyl L-carnitine (ALCAR) and a ploYclonal anti-serotonin antibody were purchased from Sigma (St. Louis, MO, USA). All other reagents used in this study were purchased from Sigma (St. Louis, MO, USA) unless mentioned otherwise. Fluorescently labeled secondary antibody (Cy3-conjugated goat anti-rabbit antibody) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

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

For treatment with ketamine and ALCAR, 52 hpf embryos were used. Fifty embryos were placed in individual 60 mm Petri dishes for each treatment. Ketamine and ALCAR treatments at various doses continued for 24 h (static exposures). Untreated control groups were examined in parallel. At 76 hpf (more than 72 hpf), zebrafish embryos are also called larvae. For consistency, we chose to use the term 'embryo' for 76 hpf zebrafish throughout this manuscript.

2.4. Determination of 5-HT and 5-HIAA by high-performance liquid chromatography with electrochemical detection (HPLC/EC)

Post-exposure to ketamine and ALCAR, zebrafish embryos (76 hpf) (50/sample) were homogenized in 100 ml of 0.2 μM perchloric acid containing 1 μM dihydroxybenzylamine (DHBA) as internal standard and centrifuged at 10,000 × *g* (10 min at 4 °C). Supernatant was removed and added to a filter tube containing a 0.2 μM membrane and spun at 4000 × *g* (4 min at 4 °C). The resulting filtrate was used for HPLC analysis of monoamines. A 25 μl aliquot was directly injected into an HPLC/EC detection system connected to BASi liquid chromatography pump (West Lafayette, IN, USA), a Supelco Supelcoil LC-18 (7.5 cm × 4.6 mm) analytical column, an amperometric detector and an LC-17 oxidative flow cell (BASi) with a glassy carbon electrode versus an Ag–AgCl reference electrode which was maintained at a potential of 0.75 V. The HPLC mobile phase consisted of potassium phosphate pH 3.0, methanol and heptane-sulfonic acid [1] and chromatograms were recorded and integrated using a BASi HPLC system with Epsilon Integrator. Concentrations of 5-HT and 5-HIAA were calculated against series of internally prepared, known standards [17]. Ratios of 5-HIAA/5-HT were calculated as estimated 5-HT release and turnover.

2.5. Immunocytochemistry for 5-HT (serotonergic) neurons in the brain

Embryos ($n = 15$/exposure group) post-exposure to ketamine and ALCAR were fixed with 4% paraformaldehyde in phosphate buffer, pH 7.3, overnight at 4 °C. Fixed embryos were washed 3 × 5 min each in phosphate buffer saline (PBS), pH 7.3 followed by permeabilization in acetone at −20 °C for 10 min. To reduce nonspecific binding by the anti-serotonin antibody, embryos were incubated in PBS plus 10% goat serum plus 1% Triton-X 100 (blocking buffer) for 1 h at room temperature. The embryos were incubated overnight at 4 °C with the anti-serotonin polyclonal antibody (1:500 dilution) in the blocking buffer. Following antibody incubation, embryos were washed 4 × 25 min each with the blocking buffer to remove excess primary antibody. Embryos were then incubated overnight at 4 °C in the blocking buffer containing Cy3-conjugated goat anti-rabbit anti-serum as the secondary antibody (1:100 dilution). Subsequent to multiple washes for 2 h in blocking buffer and a final wash with PBS for 30 min, labeled embryos were visualized and images acquired using a Nikon Eclipse Ni-U upright microscope.