Published in final edited form as:

Neurosci Lett. 2018 August 24; 682: 56–61. doi:10.1016/j.neulet.2018.06.014.

N-acetylcysteine prevents ketamine-induced adverse effects on development, heart rate and monoaminergic neurons in zebrafish

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Abstract

N-acetylcysteine, a precursor molecule of glutathione, is an antioxidant. Ketamine, a pediatric anesthetic, has been implicated in cardiotoxicity and neurotoxicity including modulation of monoaminergic systems in mammals and zebrafish. Here, we show that N-acetylcysteine prevents ketamine's adverse effects on development and monoaminergic neurons in zebrafish embryos. The effects of ketamine and N-acetylcysteine alone or in combination were measured on the heart rate, body length, brain serotonergic neurons and tyrosine hydroxylase-immunoreactive (TH-IR) neurons. In the absence of N-acetylcysteine, a concentration of ketamine that produces an internal embryo exposure level comparable to human anesthetic plasma concentrations significantly reduced heart rate and body length and those effects were prevented by N-acetylcysteine co-treatment. Ketamine also reduced the areas occupied by serotonergic neurons in the brain, whereas N-acetylcysteine co-exposure counteracted this effect. TH-IR neurons in the embryo brain and TH-IR cells in the trunk were significantly reduced with ketamine treatment, but not in the presence of N-acetylcysteine. In our continued search for compounds that can prevent ketamine toxicity, this study using specific endpoints of developmental toxicity, cardiotoxicity and neurotoxicity, demonstrates protective effects of N-acetylcysteine against ketamine's adverse effects. This is the first study that shows the protective effects of N-acetylcysteine on ketamine-induced developmental defects of monoaminergic neurons as observed in a whole organism.

Keywords

Ketamine; Zebrafish; N-acetyl cysteine; Developmental toxicity; 5-HT; Tyrosine hydroxylase

1. Introduction

N-acetylcysteine (NAC), an over-the-counter antioxidant was approved by US Food and Drug Administration (FDA) for the treatment of acetaminophen overdose in both adult and pediatric patients [1]. Ketamine, an N-methyl D-aspartate (NMDA) receptor antagonist, is an

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anesthetic [2]. Prolonged exposure to higher concentrations of ketamine during early development in mammals causes neurotoxicity [reviewed in [3]. Subanesthetic concentrations of ketamine, however, act as fast antidepressants [4]. Previously, we showed that in zebrafish embryos, ketamine adversely affected motor neurons [5], serotonergic and dopamine systems [6,7] as well as heart rate [8]. In rodents, NAC prevented ketamine-induced persistent deficits in prefrontal cortex-dependent behaviors [9], symptoms of schizophrenia [10], mitochondrial toxicity [11] and reduced GABAergic function in the prefrontal cortex [12]. NAC and ketamine prevented MPTP-induced dopaminergic neuronal death in mice [13]. However, NAC's effect on the ketamine-induced abnormal development of the monoaminergic neurons have not been shown.

The monoaminergic neurons use dopamine and serotonin (5-hydroxytryptamine or 5-HT) as neurotransmitters [14]. Dopaminergic neurons/cells express tyrosine hydroxylase (TH) [15]. Zebrafish embryos have a functional 5-HT system in the brain beginning at 24 h post-fertilization (hpf) [16] and 5-HT neurons and projections appear in the hindbrain and spinal cord at about 48 hpf [17]. To prevent or minimize ketamine toxicity that might occur from anesthesia or abuse, we are testing various compounds based on the potential mechanisms revealed in our previous studies [6,8,18–20]. One such mechanism is that since ketamine is a calcium antagonist, its toxicity could be prevented in the presence of acetyl L-carnitine (ALCAR) that is known to upregulate L-type calcium channels [21]. The concept of the ability of compounds that increase cellular calcium to prevent ketamine toxicity has not been tested. Here, we explored whether NAC, which has recently been shown to increase intracellular calcium in human neutrophils [22], would be as effective against ketamine toxicity in zebrafish.

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). Handling and maintenance of zebrafish following the NIH Guide for the Care and Use of Laboratory Animals and our Institutional Animal Care and Use Committee (IACUC) protocol are detailed in our earlier studies [23,24]. Accordingly, fertilized eggs were collected, placed in Petri dishes, 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 (10 mg/ml vials) was purchased from Vedco, Inc. (St. Joseph, MO, USA). N-acetyl cysteine (NAC) and other reagents were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. NAC stock (1 M) solutions were made fresh with buffered egg water. Rabbit polyclonal anti-Serotonin (5-HT) antibody (Cat# S5545, Sigma) and mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (Cat#MAB318, Millipore, CA, USA) were used for immunohistochemical studies. Cy3-conjugated anti-rabbit IgG and

anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.3. Treatment of zebrafish embryos with ketamine and N-acetyl cysteine (NAC)

For treatment with ketamine and NAC, 28 hpf embryos were used since our previous studies showed that these embryos as whole organisms respond to ketamine anesthesia [8,18] and ketamine exposure at the gastrula stage leads to non-specific effects [25]. For each experiment that analyzed all the endpoints, eggs laid at the same time from several females were pooled. For each treatment, 10 embryos were placed in one of the wells of the 6-well plates containing 5 ml buffered egg water. A duplicate set of treatment groups was set up in a second 6-well plate, thus for each exposure group 20 embryos were used. Treatment with drugs was blinded. At the specific experimental time points, embryos were collected for blinded examination for heart rate, body length and immunohistochemical analyses, each endpoint analysis performed by three different investigators. The experiment was repeated three times. Ketamine (2.0 mM) treatment continued for 2 h or 20 h (static exposures) in the presence or absence of NAC (0.1–1.0 mM). Previously, we have shown that the amount of ketamine accumulation (~8 μ M) in the zebrafish embryos/larvae treated with 2 mM is closer to the lower range of human anesthetic range (plasma concentration) [26]. Untreated control groups were examined in parallel.

2.4. Scoring heart rate and measuring body length

Heart rates of the embryos were scored as described in our previous studies [8,19]. The data are presented by converting heart beats per minute to percentage of control (100%). Data for each group (n = 20) were used to calculate the mean and standard deviation (SD). Embryo body lengths were measured as described in our previous study [19]. Images of the embryos were acquired using a DP2 BSW microscope (Olympus, Tokyo, Japan). Body length was quantitated using a DP2 BSW microscope digital camera software. Data for each group (n = 20) were used to calculate the mean and standard deviation (SD). The statistical significance of the effects of the various treatments on heart rate and body length was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison post-hoc analysis. Statistical significance was set at $P < 0.05$.

2.5. Whole mount immunohistochemistry

Whole mount immunohistochemistry for 5-HT (serotonergic) and dopaminergic/tyrosine hydroxylase (TH) neurons was performed as described in our previous study [7]. Briefly, for each experiment, embryos (n = 10/exposure group or control) were used. Concentrations of anti-serotonin polyclonal antibody and anti-tyrosine hydroxylase antibody were 1:500 and 1:200, respectively. 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 IgG or goat anti-mouse IgG as the secondary antibody (1:100 dilution). After 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. Using the microscope-associated image analysis software, areas in the brain, immunoreactive to 5-HT and TH antibodies, were measured from representative embryos (n = 6/treatment group) for

comparison. The area for the control/untreated embryos was considered as 100% and the changes in the treated groups were calculated accordingly. TH-immunoreactive (TH-IR) neurons in the trunk region were counted from specific areas (three hemi-segments adjacent to the distal end of the yolk extension) from representative embryos (n = 10/each treatment group) following a similar procedure we previously used to count spinal motor neurons [5]. The number of neurons in control/untreated embryos was presented as 100%, and the changes in the treated groups were calculated accordingly. Data are presented as means $\pm$ SD. The statistical significance ($P < 0.05$) of the effects induced by various treatments on 5-HT and TH-IR areas in the brain or TH-IR cells in the trunk was determined by one-way ANOVA (Sigma Stat) using Holm–Sidak pairwise multiple comparison post-hoc analysis.

3. Results

3.1. NAC prevents ketamine-induced attenuated heart rate

Zebrafish embryos at 48 hpf were exposed to 2 mM ketamine for 2 h in the presence or absence of 0.1–1 mM NAC. Ketamine caused a significant decrease in heart rate ($P < 0.001$) (Fig. 1). Ketamine-induced attenuation of heart rate was completely abolished by co-treatment with 1 mM NAC, but not with lower concentrations (0.1 and 0.5 mM) of NAC. Although in the presence of 0.5 mM NAC, ketamine's adverse effect on the heart rate was significantly increased ($P < 0.001$) compared to ketamine-treated embryos, it was still significantly lower ($P < 0.001$) than the control (Fig. 1). These results indicate that NAC prevents ketamine's adverse effects on heart rate.

3.2. Developmental toxicity by ketamine is prevented by co-treatment with NAC

Manually-dechorionated zebrafish embryos at 28 hpf were exposed to 2 mM ketamine for 20 h in the presence or absence of 1 mM NAC. Post-exposure, 48 hpf embryos showed ketamine-induced growth retardation compared to control ($P < 0.001$) (Fig. 2A, B, E). In the presence of 1 mM NAC however, ketamine was ineffective in inducing such developmental toxicity (Fig. 2C, E). There was no statistical difference in body lengths of embryos either treated with NAC (Fig. 2D, E) alone or in the presence of ketamine, suggesting that NAC can prevent ketamine-induced developmental toxicity.

3.3. Ketamine-induced toxicity of 5-HT neurons is prevented by co-treatment with NAC

Embryos at 28 hpf were treated with ketamine and NAC alone or in combination for 20 h. In the 48 hpf embryos, immunofluorescent images clearly showed serotonin-positive (5-HT) neurons in the brains (Fig. 3). Compared to the control group (Fig. 3A), embryos treated with 2 mM ketamine showed significantly reduced areas of 5-HT immunoreactivity (Fig. 3B, E). NAC in the presence of ketamine also reduced the area and intensity of 5-HT labeling (Fig. 3C, E) compared to the control but showed increased area of 5-HT-positive staining over ketamine-treated embryos (Fig. 3B, E). Treatment with NAC alone did not produce any significant changes in 5-HT immunoreactivity compared to control (Fig. 3D, E). These results suggested that NAC prevented ketamine-induced reduction in the 5-HT-positive areas in the brain.

3.4. NAC prevents ketamine's adverse effects on tyrosine hydroxylase-immunoreactive (TH-IR) neurons

Embryos at 28 hpf were treated with ketamine and NAC alone or in combination for 20 h. At 48 hpf, immunofluorescent images showed TH-IR neurons in the brains (Fig. 4). Compared to control (Fig. 4A), ketamine-treated embryos showed significantly reduced TH immunostaining patterns (Fig. 4B, E). NAC in the presence of ketamine also reduced the TH-IR area (Fig. 3C, E) compared to the control ($P < 0.001$) but showed significantly increased area of TH-positive staining over ketamine-treated embryos ($P < 0.001$) (Fig. 3B, E). Treatment with NAC alone did not have any effect on the TH-IR areas (Fig. 4A, D, E). These results suggested that NAC, to some extent, prevented ketamine-induced reduction in the TH-IR areas in the brain.

3.5. NAC prevents ketamine-induced reduction in trunk TH-IR cells

In zebrafish embryos, anti-TH immunoreactivity just ventral to the dorsal aorta reveals sympathetic neurons [27]. Our data show that compared to control (Fig. 5A, E), the number of TH-IR cells was significantly reduced in 2 mM ketamine-treated embryos (Fig. 5B, E). TH-IR cell size was also reduced in ketamine-treated embryos. However, co-treatment with 1 mM NAC prevented ketamine's adverse effects (Fig. 5C, E). NAC alone did not appear to have any effect on the trunk TH-IR neurons (Fig. 5D, E). These results suggest that NAC can prevent ketamine-induced adverse effects on the trunk TH-IR cells.

4. Discussion

The current study shows that NAC can counteract ketamine-induced cardiotoxicity, developmental toxicity and monoaminergic neuron toxicity. Ketamine toxicity, such as cardiotoxicity [23] and neurotoxicity [reviewed in [3]] in mammals have been reported. Ketamine exposure in humans can cause intrauterine growth retardation [28]. Our studies show that 1 mM NAC could effectively prevent ketamine-induced growth retardation and attenuated heart rate. NAC at 1 mM has been shown to partially prevent rotenone-induced cell death in the larval zebrafish brain [29].

In chronic ketamine users, the prefrontal dopaminergic system is adversely affected [30]. We show that at 48 hpf, 5-HT is expressed in the neurons in the lower part of the diencephalon as opposed to an expanded expression in the diencephalon that we reported in 3-day old larvae [7]. NAC prevented the reduction in the area occupied by 5-HT neurons seen in ketamine-treated embryos indicating that NAC can prevent ketamine's adverse effects on the 5-HT neuron development. In zebrafish, 5-HT is expressed in sympathetic neurons and neuroepithelial cells on the skin and trunk [31]. We observed non-specific background staining in the zebrafish embryo trunk (data not shown) with the antibody we used. Therefore, it was difficult to determine what effects ketamine and NAC have on those cells. We have previously shown functional evidence that ALCAR's ability to open L-type calcium channels could be at play to compensate for ketamine's blockade of calcium-permeable NMDA receptors, especially during prolonged exposures [8,19,25]. Interestingly, NAC has been shown to increase intracellular calcium [22]. Therefore, we speculate that

NAC's mode of action in preventing ketamine toxicity of 5-HT neurons in the zebrafish embryos may rely on the intracellular calcium levels.

Ketamine caused significant reduction in TH-IR neurons in the zebrafish embryo brain, suggesting that ketamine either directly inhibited dopaminergic neuron development or the reduction in TH-IR area is a result of an overall developmental delay caused by ketamine. Previously, we showed that ketamine reduced dopamine levels in 76 hpf larvae [6]. TH expression was shown to increase in the zebrafish larvae after treatment with 0.1 μ M ketamine, but not with 10 or 50 μ M ketamine added to the embryo water [32]. Since we used 2 mM ketamine that results in an internal exposure of $\sim$ 8 μ M [26], it is not possible to compare our results with those. In ketamine-treated monkeys, reduced dopamine levels in the prefrontal cortex [33] suggests that ketamine could adversely affect TH-IR neurons as observed in the zebrafish embryos exposed to ketamine.

TH-positive cells are present in the spinal cord of zebrafish larva [34]. Both TH and 5-HT reactivity were found in ventral spinal cord of zebrafish larvae, where amines could potentially interact directly with motor neurons [17]. In the 48 hpf zebrafish embryo, TH-positive cells are present in the sympathetic ganglia of the spinal cord [27]. The current study showed that TH-IR cells were present adjacent to the spinal cord of the 48 hpf embryos. Ketamine significantly reduced the number and size of these TH-IR cells. But in the presence of NAC, ketamine's adverse effects were prevented. NAC alone did not seem to affect the trunk TH-IR cells.

Since NAC possesses both antioxidative and pro-oxidative properties [35], the status of ROS in ketamine-induced toxicities and the mechanism of their prevention by NAC would require further investigation. In summary, NAC prevented ketamine-induced multiple toxicities and the findings will be beneficial in understanding the nature of drug toxicities and potential treatments in humans.

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Fig. 1.

NAC prevents ketamine's adverse effects on heart rate.

Embryos (48 hpf) were exposed for 2 h to ketamine (2 mM) alone or in the presence of various concentrations of NAC and 1 mM NAC alone. Heart rate was measured as described in the Materials and methods section. Statistical significance (*) was set at $P < 0.05$.

Fig. 2.

NAC prevents ketamine's adverse effects on growth.

Zebrafish embryos at 28 hpf (manually dechorionated) were exposed for 20 h to drugs.

Linear body lengths of the untreated control (A) and embryos treated with 2 mM ketamine (B), 2 mM ketamine + 1 mM NAC (C) and 1.0 mM NAC alone, were compared as percentage change over control (E). Statistical significance is indicated (*).

Fig. 3.

NAC prevents ketamine's adverse effects on the 5-HT neurons.

Zebrafish embryos at 28 hpf (manually dechorionated) were exposed for 20 h to drugs.

Anti-5-HT-immunostained embryos at 48 hpf are shown. Anterior is upwards (A–D). Eyes (e) and yolk sac are indicated. Brain areas with 5-HT neurons in control (A); 20 h post-exposure to 2 mM ketamine (B), 2 mM ketamine + 1 mM NAC (C) and 1 mM NAC (D) are shown. Quantification of relative areas occupied by 5-HT neurons in the experimental groups are presented (E). Asterisk indicates significant difference.

Fig. 4.

NAC counteracts ketamine-induced TH-IR neuron toxicity in the brain.

Anti-TH antibody-stained whole mount immunohistochemistry of the anterior part of the embryos at 48 hpf are shown. Anterior is upwards (A–D). Eyes (e) and yolk sac are indicated. Specification of midbrain TH-IR neurons in the 48 hpf embryos, demarcated by dotted bracket lines in control (A); 20 h post-exposure to 2 mM ketamine (B), 2 mM ketamine + 1 mM NAC (C) and 1 mM NAC (D). Relative areas occupied by TH-IR neurons in each experimental group are presented (E). Asterisk indicates significant difference.

Fig. 5.

NAC restores ketamine-induced reduction in TH-IR cells in the trunk.

Anti-TH antibody-stained trunk regions (lateral view) of the 48 hpf embryo control (A), 20 h exposure at 28 hpf to 2 mM ketamine (B), 2 mM ketamine + 1 mM NAC (C) and 1 mM NAC alone (D). Anterior is to the left and posterior is to the right (A–D). Yolk extension (YE) and some TH-IR cells (arrows) are indicated. Quantification of relative numbers of TH-IR cells in each experimental group are presented (E). Asterisk indicates a significant difference.