Published in final edited form as:

Neurotoxicol Teratol. 2016 ; 54: 52–60. doi:10.1016/j.ntt.2016.02.004.

Distinct effects of ketamine and acetyl L-carnitine on the dopamine system in zebrafish

Bonnie L. Robinson, Melanie Dumas, Elvis Cuevas, Qiang Gu, Merle G. Paule, Syed F. Ali, and Jyotshna Kanungo*

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

Abstract

Ketamine, a noncompetitive *N*-methyl-D-aspartic acid (NMDA) receptor antagonist is commonly used as a pediatric anesthetic. We have previously shown that acetyl L-carnitine (ALCAR) prevents ketamine toxicity in zebrafish embryos. In mammals, ketamine is known to modulate the dopaminergic system. NMDA receptor antagonists are considered as promising anti-depressants, but the exact mechanism of their function is unclear. Here, we measured the levels of dopamine (DA) and its metabolites, 3, 4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in the zebrafish embryos exposed to ketamine in the presence and absence of 0.5 mM ALCAR. Ketamine, at lower doses (0.1–0.3 mM), did not produce significant changes in DA, DOPAC or HVA levels in 52 h post-fertilization embryos treated for 24 h. In these embryos, tyrosine hydroxylase (TH) mRNA expression remained unchanged. However, 2 mM ketamine (internal embryo exposure levels equivalent to human anesthetic plasma concentration) significantly reduced DA level and TH mRNA indicating that DA synthesis was adversely affected. In the presence or absence of 2 mM ketamine, ALCAR showed similar effects on DA level and TH mRNA, but increased DOPAC level compared to control. ALCAR reversed 2 mM ketamine-induced reduction in HVA levels. With ALCAR alone, the expression of genes encoding the DA metabolizing enzymes, MAO (monoamine oxidase) and catechol-*O*-methyltransferase (COMT), was not affected. However, ketamine altered MAO mRNA expression, except at the 0.1 mM dose. COMT transcripts were reduced in the 2 mM ketamine-treated group. These distinct effects of ketamine and ALCAR on the DA system may shed some light on the mechanism on how ketamine can work as an anti-depressant, especially at sub-anesthetic doses that do not affect DA metabolism and suppress MAO gene expression.

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

Disclaimer

This document has been reviewed in accordance with 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.

Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

Keywords

Ketamine; Zebrafish; Acetyl L-carnitine; Dopamine; Tyrosine hydroxylase; MAO

1. Introduction

The pediatric general anesthetic, ketamine, is a noncompetitive antagonist of the *N*-methyl-D-aspartic acid (NMDA) receptor (Kohrs and Durieux, 1998). Although recognized as a dissociative anesthetic (Corssen et al., 1968), ketamine also has analgesic and amnesic effects and its potential anti-depressant properties are also being investigated (Aroni et al., 2009; Berman et al., 2000; Niesters et al., 2012; Rowland, 2005). Ketamine is listed as a Schedule III controlled substance in the USA, due to its potential for abuse (Morgan et al., 2010; Rowland, 2005). A number of studies on rodents and nonhuman primates have shown that treatment with high doses of ketamine or exposure for long durations during susceptible periods of development (the brain growth spurt) can induce abnormally high levels of neuroapoptosis (Haberny et al., 2002; Ikonomidou et al., 1999; Lahti et al., 2001; Larsen et al., 1998; Malhotra et al., 1997; Maxwell et al., 2006; Slikker et al., 2007; Wang et al., 2006).

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 during ATP generation (Bieber, 1988). ALCAR effectively prevents mitochondrial injury resulting from oxidative damage (Palacios et al., 2011). It is also suggested that the carnitines have neuroprotective effects on conditions associated with mitochondrial dysfunction and oxidative stress and possibly in neurodegenerative disorders such as Parkinson's disease (Beal, 2003). During early developmental stages, co-administration of ALCAR significantly diminished reactive oxygen species (ROS) generation and provided significant protection of neurons from ketamine-induced neurodegeneration in rats (Liu et al., 2013). Additionally, ALCAR has been shown to protect neurons from inhalation anesthetic-induced neurotoxicity in rat cortical neurons (Zou et al., 2008). In zebrafish embryos, ALCAR offers protection against ketamine-induced cardiotoxicity and neurotoxicity (Cuevas et al., 2013; Kanungo et al., 2012). ALCAR reversed the effects of 2 mM ketamine not only by restoring serotonin (5-HT) and its metabolite (5-hydroxyindoleacetic acid or 5-HIAA) levels but also 5-HT turnover rate to control levels in the zebrafish embryos (Robinson et al., 2015).

In the dopamine (DA) biosynthesis process, conversion of L-tyrosine to L-DOPA by tyrosine hydroxylase (TH) is the rate-limiting step (Kuhn et al., 1999). DOPA is subsequently converted to DA by L-aromatic amino acid decarboxylase. Released DA is metabolized to 3,4-dihydroxyphenyl-acetic acid (DOPAC) by intra-neuronal monoamine oxidase (MAO) after reuptake by the nerve terminal and is also converted to 4-hydroxy-3-methoxyphenylacetic acid (HVA), probably at an extra-neuronal site through the sequential action of catechol-*O*-methyltransferase (COMT) and MAO (Meiser et al., 2013). A number of studies suggest that ketamine not only affects the glutamate and GABA system (Boczek et al., 2015; Catena-Dell'Osso et al., 2013) but also may influence the DA system (Li et al., 2015; Owolabi et al., 2008; Tan et al., 2012) in a way that could contribute to the disruption

of normal development. Ketamine reportedly exhibits a strong affinity for the D2 dopaminergic receptors indicating that the DA system could be modulated by ketamine (Kapur and Seeman, 2001). The prefrontal dopaminergic system, responsible for working memory and executive function, appears to be adversely affected by chronic ketamine exposure (Narendran et al., 2005). In young monkeys anesthetized during the brain growth spurt, ketamine has been shown to cause long-lasting deficits in several cognitive functions (Paule et al., 2011). Pharmacological evidence suggests that NMDAR dysfunction may cause dopaminergic dysfunction in schizophrenia (Rujescu et al., 2006; Stefani and Moghaddam, 2005) and ketamine has been shown to induce DA release in lateral prefrontal and anterior cingulate cortex in healthy men (Aalto et al., 2005). However, little is known about the precise mechanism of ketamine's effects on the DA system.

In MPTP-treated monkeys, ALCAR provided behavioral protection from MPTP, but it also altered DA mechanisms since HVA values were about 30% of those of normal monkeys in the caudate nucleus and about 60% in the substantia nigra (Bodis-Wollner et al., 1991). A week-long regimen of ALCAR treatment produced a steady increase in DA and serotonin (5-HT) output in the nucleus accumbens shell in rats (Tolu et al., 2002) and striatal infusion of ALCAR increased DA efflux with no changes in the efflux of DOPAC or HVA (Harsing et al., 1992). A 4-day treatment with ALCAR elevated GABA levels in rat substantia nigra but not striatal DA (Fariello et al., 1988). ALCAR reportedly attenuated age-related changes in dopaminergic systems in rats (Sershen et al., 1991). Since ALCAR did not show any normalizing effects on catecholamine concentrations, TH, neurofilament and glial fibrillary acid protein, it has been suggested that ALCAR's protective role may be related to energy metabolism and not to direct effects on the dopaminergic systems (Pettegrew et al., 2000; Santarelli et al., 1995; Steffen et al., 1995). Chronic administration of ALCAR has been shown to lead to enhanced DA release in the striatum (Sershen et al., 1991).

In addition to its small size, prolific reproductive capacity, and easy maintenance, the zebrafish possesses the typical complexity of vertebrate systems and therefore has been used in several areas of research with the prospect of extrapolating findings to mammals including humans (Briggs, 2002; Kanungo et al., 2014; Lantz-McPeak et al., 2015; Parnig et al., 2002). Since its early use, emphasis has been given to characterizing molecules within the zebrafish nervous system, a difficult task to undertake in mammals (Key and Devine, 2003). Zebrafish embryos/larvae have a functional DA system and they accordingly respond to a number of drugs including drugs of abuse (Buske and Gerlai, 2011; Kung et al., 2015; Levin et al., 2011; Suen et al., 2013). Furthermore, zebrafish has been used to study the antidepressant-like effects of MK-801, an NMDA receptor antagonist (da Silva et al., 2015). Our earlier studies demonstrated that exposure of embryos to 2 mM ketamine resulted in an internal exposure of ~8 μ M (Trickler et al., 2014). This concentration is comparable to the lower end of the human anesthetic blood levels (2.2 μ g/ml). Since ketamine is lately being used as a fast-acting anti-depressant (Bliser, 2013), in the present study, in order to gain insight into the mechanism of ketamine's effect on the DA system, we examined whether ketamine and ALCAR evoke a response in the DA system in the zebrafish embryos in terms of DA biosynthesis, metabolism, and change the expression of in TH, MAO and COMT genes.

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 liter tank housed 8 adult males or 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 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) was 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.

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

For treatment with ketamine and ALCAR, 52 h post-fertilization (hpf) embryos were used. For each treatment 50 embryos were placed in individual 60 mm Petri dishes containing 35 ml buffered egg water. For each treatment, replicates were set up with a total of 150 embryos in three 60 mm Petri dishes. Ketamine (0.1, 0.2, 0.3 and 2.0 mM) and ALCAR (0.5 mM) treatments, alone or in combination continued for 24 h (static exposures). Untreated control groups were examined in parallel. ALCAR (0.5 mM) treatments were for 20 and 24 h (static exposure). At 72 or 76 hpf, zebrafish embryos are also called larvae. For consistency, we use the term ‘embryo’ throughout this manuscript.

2.4. High-performance liquid chromatography with electrochemical detection (HPLC/EC)

Post-exposure to ketamine and ALCAR, 76 hpf zebrafish embryos (50/sample, $n = 3$ for each sample) were homogenized in 100 μ l of 0.2 μ M perchloric acid containing 1 μ M dihydroxybenzylamine (DHBA) as internal standard and centrifuged at $10,000 \times g$ (10 min at 4 °C). Supernatant was removed and added to a filter tube containing a 0.2 μ M membrane and spun at $4000 \times g$ (4 min at 4 °C). The resulting filtrate was used for HPLC analysis of monoamines. A 25 μ l aliquot from each sample was directly injected into an HPLC/EC system connected to BASi liquid chromatography pump (West Lafayette, IN, USA), a Supelco Supelcoil LC-18 (7.5 cm $\times$ 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 (Ali et al., 1993) and chromatograms were recorded and integrated using a BASi HPLC system with Epsilon Integrator. Concentrations of DA, DOPAC and HVA were calculated against a series of internally prepared, known standards and ratios of DOPAC/DA and HVA/DA were calculated as estimates of DA release and turnover (Boireau et al., 1990; Hamilton et al., 2012).

2.5. RNA extraction and cDNA synthesis

Total RNA (pooled from 50 embryos/treatment group) was extracted from whole embryos using Trizol reagent (Invitrogen, Carlsbad, CA, USA). An aliquot of each RNA sample was used to spectrophotometrically determine RNA quality and concentration using a NanoDrop ND-2000 (NanoDrop Technology, Wilmington, DE, USA) ($A_{260}/A_{280} > 2.0$). First-strand cDNA was synthesized from total RNA (2 µg; 20 µl final reaction volume) with oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

2.6. Primers

The following primers were used for the quantitative polymerase chain reaction (qPCR) assays: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-GATACACGGAGCAC CAGGTT-3' and reverse 5'-GCCATCAGGTCACATACACG-3'; tyrosine hydroxylase (TH) forward 5'-CAAGCAGCTCCACATCTTCC-3' and reverse 5'-CATCGCTCTCCTCAA ACACG-3'; monoamine oxidase (MAO) (GenBank Accession # BC070013.1) forward 5'-ACCAACTCAAACCGCATTC-3' and reverse 5'-GTAGGCAAAAGGGTTCCACA-3'; catechol-*O*-methyltransferase (COMT) (GenBank Accession # HM997189.1) forward 5'-CGGCTCCTGAACCTTCT TCAC-3' and reverse 5'-GTGCTGGGATTACCTCTGT-3'.

2.7. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was performed using a CFX96 C1000 (Bio-Rad, Hercules, CA, USA) detection system with SYBR green fluorescent label (Bio-Rad). Samples (25 µl final vol) contained the following: 1 × SYBR green master mix (Bio-Rad), 5 pmol of each primer, and 0.25 µl of the reverse transcriptase (RT) reaction mixture. Samples were run in triplicate in optically clear 96-well plates. Cycling parameters were as follows: 50°C × 2 min, 95°C × 10 min, then 40 cycles of 95°C × 15 s, 60°C × 1 min. A melting temperature-determining dissociation step was performed at 95°C × 15 s, 60°C × 15 s, and 95°C × 15 s at the end of the amplification phase. The GAPDH gene was the internal control for the qRT-PCR experiments. Negative controls included reactions without the cDNA template or cDNA reaction products without the reverse transcriptase. Specificity of amplification was confirmed by the melting curve with one peak in PCR reactions.

2.8. Statistical analyses

In order to determine DA, DOPAC and HVA levels, data from biological replicates were averaged. For the qRT-PCR data, the 2^{-Ct} method was used to determine the relative gene expression (Livak and Schmittgen, 2001). Data from each group were averaged and shown as normalized gene expression $\pm$ SD. For the ANOVA with Holm–Sidak pair-wise multiple comparison post-hocs, different treatment groups were used as independent variables while dependent variables were the experimental outcomes. Statistical significance was set at $P = <0.05$.

3. Results

3.1. Low doses (0.1–0.3 mM) of ketamine do not alter DA levels

Zebrafish embryos at 52 hpf were exposed to 0.1–0.3 mM ketamine for 24 h. We observed that 0.1–0.3 mM ketamine did not induce complete anesthesia since the embryos were partially mobile. None of three doses (0.1, 0.2 and 0.3 mM) altered DA levels when compared to the control (Fig. 1A). Levels of DOPAC and HVA, metabolites of DA, also did not change (Fig. 1B, C) indicating that, 24 h of exposure over this dose range of ketamine does not have any effect on the zebrafish embryo DA system.

3.2. Ketamine at a dose (2 mM) equivalent to human anesthetic levels reduces DA levels

Embryos treated with 2 mM [absorption comparable to human anesthetic blood levels (2.2 μ g/ml)] had significantly reduced DA levels compared to controls (Fig. 2A). However, DOPAC levels did not change significantly in these embryos (Fig. 2B), whereas levels of HVA were significantly decreased (Fig. 2C).

3.3. ALCAR upregulates DA metabolism in the presence or absence of ketamine

Zebrafish embryos treated with 2 mM ketamine plus 0.5 mM ALCAR for 24 h exhibited significantly-reduced levels of DA compared to controls and to ketamine-treated embryos (Fig. 2A). ALCAR alone also reduced DA levels compared to the controls, embryos treated with ketamine only, and those treated with ketamine plus ALCAR (Fig. 2A).

Although ketamine at both sub-anesthetic (0.1–0.3 mM) and anesthetic (2 mM) doses did not affect DOPAC levels, ketamine plus ALCAR or ALCAR alone significantly increased DOPAC levels compared to both the control and ketamine-treated embryos (Fig. 2B). In embryos treated with 2 mM ketamine, HVA level was significantly lower than control (Fig. 2C). ALCAR and ketamine co-treatment or ALCAR alone significantly improved HVA levels in the embryos although the levels were still significantly reduced than control (Fig. 2C). The DOPAC/DA ratio was used to determine the rate of DA metabolism to DOPAC. None of the doses of ketamine utilized affected DA metabolism in the embryos (Fig. 3A, B). However, ALCAR significantly increased the rate of metabolism of DA to DOPAC more than for control or ketamine-treated embryos (Fig. 3B). These results indicated that ALCAR produces a positive effect on DA's metabolism to DOPAC in zebrafish embryos.

The rate of DA metabolism to HVA was determined by obtaining the ratios of HVA/DA levels. Ketamine did not affect DA conversion to HVA at any of the doses tested (Fig. 3C,

D). In embryos treated with ALCAR alone or with ALCAR plus ketamine, the HVA/DA ratios were significantly higher compared to those from control or ketamine-treated embryos (Fig. 3D) indicating that ALCAR accelerated DA metabolism to HVA.

3.4. Ketamine and ALCAR alter tyrosine hydroxylase (TH) gene expression

TH is the rate limiting enzyme in DA biosynthesis. Since 2 mM ketamine as well as ALCAR alone decreased DA levels in the zebrafish embryos, it was necessary to determine whether DA biosynthesis was compromised at the transcriptional level. Data obtained using qRT-PCR indicated that low doses (0.1–0.3 mM) of ketamine did not alter TH mRNA expression (Fig. 4A). DA levels were also not altered in these embryos (Fig. 1A). However, the anesthesia-relevant dose of ketamine (2 mM) significantly reduced TH mRNA expression as did ALCAR alone or ALCAR with ketamine (Fig. 4B). These data suggest that the lower DA levels observed in these groups (Fig. 2A) result from reduced TH mRNA expression.

3.5. Effects of ketamine and ALCAR on the DA metabolizing enzyme gene expression

In order to determine whether the effects of ketamine and ALCAR on DA metabolism could result from the altered gene expression of the DA metabolizing enzymes, monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) (Fig. 5A), we performed RT-qPCR. Ketamine at lower doses (0.1–0.3 mM) did not alter COMT gene expression, while 0.2 and 0.3 mM ketamine significantly down regulated MAO gene expression (Fig. 5B).

A higher dose of ketamine (2 mM), in the presence or absence of ALCAR, down-regulated both MAO and COMT gene expression (Fig. 5C). However, ALCAR alone did not have any effect on the expression of both MAO and COMT genes (Fig. 5C). At lower doses (0.05, 0.15 and 0.25 mM), ALCAR did not alter MAO and COMT gene expression (data not shown). In co-exposure experiments, ALCAR's presence appeared to have modestly reversed the suppressive effects of ketamine on COMT mRNA expression, but did not have any positive effect on ketamine-triggered suppression of MAO transcripts (Fig. 5C). These results indicate that compared to control, ALCAR does not alter the MAO and COMT gene expression, while ketamine reduces MAO at low and high doses and COMT mRNA at high dose only.

4. Discussion

Ketamine's toxic effects on zebrafish embryos and larvae have been well documented (Cuevas et al., 2013; Felix et al., 2014; Kanungo et al., 2013; Kanungo et al., 2012; Lantz-McPeak et al., 2015; Riehl et al., 2011). We have previously shown that ALCAR reverses ketamine's neurotoxic and cardiotoxic effects on zebrafish embryos (Cuevas et al., 2013; Kanungo et al., 2012) and enhances 5-HT metabolism (Robinson et al., 2015). Ketamine's use as a rapid acting antidepressant (Blier, 2013) indicates its potential effects on the monoamine neurotransmitters, such as dopamine (DA), although the exact mechanism is not clear. Since zebrafish embryo model is an ideal system for mechanistic studies, we investigated whether ALCAR and ketamine differentially modulated the DA system in zebrafish embryos. The expression of TH was measured to determine if developmental ketamine and ALCAR exposure adversely affected DA biosynthesis at the transcriptional

level of this rate-limiting enzyme. Lower doses of ketamine (0.1–0.3 mM) did not have any effect on DA levels or TH mRNA. Ketamine, at 0.1 and 1.0 μ M but not at 10 or 50 μ M, doses has been shown to induce TH expression in zebrafish larvae (Suen et al., 2013). However, at 10 or 50 μ M concentrations, ketamine's inability to alter TH and DA levels was attributed to the induction of anesthesia in zebrafish (Suen et al., 2013). In our current study, we observed that 0.1–0.3 mM ketamine did not induce complete anesthesia in the embryos and these doses did not alter DA levels. In patients, ketamine plasma sub-anesthetic concentrations range from 0.5–1 μ M and anesthesia sets in at ~10–100 μ M range (Domino et al., 1982; Geisslinger et al., 1993; Idvall et al., 1979). Our studies on zebrafish embryos treated with 2 mM ketamine that were completely anesthetized (Cuevas et al., 2013) show an internal exposure of ~8 μ M (Trickler et al., 2014) and is comparable to the human anesthetic dose range. Although NMDA receptor blockade can indirectly lead to DA release (Svensson et al., 1995; Zhang et al., 1993), ketamine treatment did not change DA levels in mice (Irifune et al., 1997; Kelland et al., 1990), or striatal DA metabolism in rats for up to 9 h post-injection (Koshikawa et al., 1988). Our results showing no effect of ketamine, at 0.1–0.3 mM doses, on the DA system are in agreement with these mammalian data. In consistence, even at 2 mM, ketamine did not induce alteration to DA metabolism although it significantly reduced DA levels in the zebrafish embryos compared to the control.

It has been shown that ketamine (3–150 mg/kg) dose-dependently induces hyper-locomotion in mice (Irifune et al., 1991). On the other hand, in the higher dose range, ketamine also induces anesthesia, and hyper-locomotion occurs only after the anesthetic phase and electrophysiological studies have shown that ketamine-induced hyperlocomotion is linked to increased DA turnover (Irifune et al., 1991). In rodents, ketamine has been shown to increase DA turnover in the nucleus accumbens (Irifune et al., 1997; Irifune et al., 1991) and the striatum (Irifune et al., 1997; Irifune et al., 1991; Ylitalo et al., 1976). In contrast, TH levels were found to be lower in the prefrontal cortex of ketamine-treated monkeys, which matched reduced DA levels in the prefrontal cortex (Yu et al., 2012). These mammalian data and the results of the present study indicate that treatment dose and duration may be responsible for varying DA levels in response to ketamine.

Although, there were no significant differences in DA, DOPAC and HVA levels or the metabolite/DA ratios between the control and low dose ketamine-treated embryos, embryos exposed to 2 mM ketamine exhibited significantly reduced DA. Our data on these neurotransmitter levels are derived from whole body preparations as opposed to very specific organs, such as the brain. It is important to note that half of the DA in human body is synthesized in the gastrointestinal tract, thus indicating DA production via a non-neuronal dopaminergic system (Eisenhofer et al., 1997). Nonetheless, HVA level in CSF, plasma and urine is considered a biomarker of CNS dopaminergic dysfunction in humans (Eisenhofer et al., 1997). Very little is known about DA metabolism in the zebrafish embryos/larvae and more research would be necessary to determine the preferred pathway of DA metabolism.

ALCAR has been shown to enhance methamphetamine-induced DA depletion in PC12 cells (Virmani et al., 2003) and chronic administration of ALCAR has been shown to enhance DA release in the striatum (Sershen et al., 1991). DA release enhances its own metabolism. In our studies, developmental exposure to ALCAR, alone or in the presence of ketamine

resulted in decreased DA levels but increased levels of DOPAC in the embryos. These reduced DA levels could be due to its enhanced metabolism. In the present study, the increased DOPAC/DA and HVA/DA ratios along with decreased DA levels in ALCAR-treated embryos indicate an altered DA system. Together, the data suggest that although DA levels were reduced in the embryos treated with either ketamine or ALCAR, it is possible that the pathways triggered by each of these two compounds leading to such reduction could be different, ketamine having a direct inhibitory effect on DA synthesis and ALCAR reducing DA levels by accelerating its metabolism.

ALCAR prevents age-related loss of NMDA receptors in the hippocampus, striatum, and frontal cortex of rats (Castorina and Ferraris, 1994) and releases DA in the rat corpus striatum via calcium-dependent exocytosis (Harsing et al., 1992). In monkeys, ALCAR protects against MPTP-induced neurotoxicity although HVA levels in animals treated with ALCAR were at about 30% of those of controls in the caudate nucleus and about 60% in the substantia nigra; without ALCAR, HVA values were 16% and 40% of control, respectively, suggesting that ALCAR did have protective actions on the dopaminergic system (Bodis-Wollner et al., 1991). Surprisingly, ALCAR-treated monkeys also showed reduced numbers of TH-labeled neurons in the substantia nigra compared to control animals, whereas in the MPTP-treated monkeys with no ALCAR treatment, there was profound loss of TH-positive neurons (Bodis-Wollner et al., 1991). In conformity, our previous studies showed that ALCAR prevented ketamine-induced neurotoxicity (Cuevas et al., 2013) and our present study shows that ALCAR with or without ketamine reduced TH gene expression.

There are two *TH* homologs (*TH1* and *TH2*) in zebrafish. Based on sequence homology, one of the two *TH* homologs, *TH2* (GenBank Accession # NM_001001829) has been identified and characterized to encode a protein with tryptophan hydroxylase activity but not TH activity (Ren et al., 2013). Therefore, we quantified their RNA expression for *TH1* (GenBank Accession # BC163630). Induction of TH via the MAPK/ERK pathway has been reported (McMillan et al., 2007). Ketamine (2 mM) has been shown to attenuate MAPK/ERK activity in the zebrafish embryos (Kanungo et al., 2012; Trickler et al., 2014). It is likely that reduced TH mRNA expression caused by 2 mM ketamine results from attenuated MAPK/ERK activity. However, that does not explain why ALCAR (with or without ketamine) which normalizes MAPK/ERK activity (Kanungo et al., 2012) also reduces TH mRNA expression. Feedback inhibition of TH by the end-product DA during DA synthesis has been described (Kumer and Vrana, 1996). In vitro and in vivo data suggest that the net effect of increased efficiency of conversion of L-DOPA to DA and feedback inhibition can lead to overall decreased DA level and TH gene expression (Chang et al., 2002). Regulation of TH is complex and control of its expression by transcriptional and post-translational mechanisms is an active field of research (Daubner et al., 2011; Doskeland and Flatmark, 2002). Since most of our studies were conducted at one time point (24 h exposure), it was difficult to know whether ALCAR initially upregulated DA synthesis via higher TH mRNA expression prior to a subsequent decrease in DA and TH mRNA expression due to feedback inhibition. Our preliminary study showed that in fact, a 20-h exposure of 52 h embryos to 0.5 mM ALCAR did induce TH mRNA expression by 1.23 fold, a modest but significant ($P < 0.05$) up regulation, while lower doses of ALCAR (0.05, 0.15 and 0.25 mM) did not have any effect (data not shown). Whether this 4 h difference in

the age of the embryos reflected a less developed DA system and hence contributed to such a contrast in TH mRNA expression or overexpression of TH followed by increased DA itself contributed to a feedback loop toward suppressing the TH gene expression, needs careful design of experiments, preferably in an in vivo setting using embryos expressing the TH-promoter driven reporter.

In mammalian cells, ALCAR does not change MAO activity (Snyder et al., 1990) and MAPK upregulates MAO gene expression (Wong et al., 2002). In zebrafish embryos, while 2 mM ketamine down-regulates ERK/MAPK, ALCAR does not have any effect (Kanungo et al., 2012). Our current study shows ketamine significantly down-regulated MAO transcripts at 0.2, 0.3 and 2.0 mM. However, at these doses DOPAC levels were unchanged, while HVA was reduced only in the 2 mM ketamine group. In mice, chronic ketamine treatment diminished MAO activity in the striatal region (Chatterjee et al., 2012). Down-regulation of MAO by ketamine may result in reduced DA metabolism thus supporting an anti-depressant function. In the zebrafish embryos, ALCAR alone did not have any effect on the MAO gene expression, despite higher levels of DOPAC in the presence or absence of 2 mM ketamine. We have previously shown that 2 mM ketamine induces testosterone levels in zebrafish embryos (Trickler et al., 2014), which is of relevance since increased testosterone attenuated MAO level in rhesus monkeys (Redmond et al., 1976). Ketamine at 2 mM, in the presence or absence of ALCAR, significantly reduced COMT expression while ALCAR alone did not have any effect. Overall, the MAO and COMT mRNA expression, in our study, did not always directly correlate with the metabolite levels indicating that post-translational modifications in regulating the enzyme activities could be involved. MAO mRNA expression not correlating with protein or gene function has been reported (Ahmed et al., 2014; Fitzgerald et al., 2007). Increase in MAO activity is selectively enhanced by Ca^{2+} in mouse, rat, monkey and human brains (Cao et al., 2009; Egashira et al., 2003; Kosenko et al., 2003; Samantaray et al., 2003). Ketamine as an antagonist of Ca^{2+} -permeable NMDA receptors, can potentially reduce MAO activity by reducing MAO mRNA expression, as observed in our current study. Nonetheless, sub-anesthetic doses of ketamine reducing MAO mRNA expression and not altering DA levels in the zebrafish embryos support the fact that sub-anesthetic doses are effective as fast-acting antidepressants in humans (Blier, 2013). In order to precisely understand their roles as anti-depressants, future studies will need to focus on whether ketamine and ALCAR differentially modulate MAO and COMT activities, and affect the development and integrity of the dopaminergic neurons.

Abbreviations

COMT	catechol- <i>O</i> -methyl transferase
DA	dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
HVA	homovanillic acid
MAO	monoamine oxidase
3-MT	3-methoxytyramine

References

- Aalto S, Ihalaenen J, Hirvonen J, Kajander J, Scheinin H, Tanila H, Nagren K, Vilkmann H, Gustafsson LL, Syvalahti E, et al. Cortical glutamate-dopamine interaction and ketamine-induced psychotic symptoms in man. *Psychopharmacology*. 2005; 182:375–383. [PubMed: 16001106]
- Ahmed AA, Ma W, Ni Y, Zhou Q, Zhao R. Embryonic exposure to corticosterone modifies aggressive behavior through alterations of the hypothalamic pituitary adrenal axis and the serotonergic system in the chicken. *Horm. Behav.* 2014; 65:97–105. [PubMed: 24333411]
- Ali SF, David SN, Newport GD. Age-related susceptibility to MPTP-induced neurotoxicity in mice. *Neurotoxicology*. 1993; 14:29–34. [PubMed: 8361676]
- Aroni F, Iacovidou N, Dontas I, Pourzitaki C, Xanthos T. Pharmacological aspects and potential new clinical applications of ketamine: reevaluation of an old drug. *J. Clin. Pharmacol.* 2009; 49:957–964. [PubMed: 19546251]
- Beal MF. Bioenergetic approaches for neuroprotection in Parkinson's disease. *Ann. Neurol.* 2003; 53(Suppl. 3):S39–S47. (discussion S47–8). [PubMed: 12666097]
- Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH. Antidepressant effects of ketamine in depressed patients. *Biol. Psychiatry*. 2000; 47:351–354. [PubMed: 10686270]
- Bieber LL. Carnitine. *Annu. Rev. Biochem.* 1988; 57:261–283. [PubMed: 3052273]
- Blier P. Exploiting N-methyl-D-aspartate channel blockade for a rapid antidepressant response in major depressive disorder. *Biol. Psychiatry*. 2013; 74:238–239. [PubMed: 23885752]
- Boczek T, Lisek M, Ferenc B, Wiktorska M, Ivchevska I, Zylinska L. Region-specific effects of repeated ketamine administration on the presynaptic GABAergic neurochemistry in rat brain. *Neurochem. Int.* 2015; 91:13–25. [PubMed: 26492822]
- Bodis-Wollner I, Chung E, Ghilardi MF, Glover A, Onofrij M, Pasik P, Samson Y. Acetyl-levo-carnitine protects against MPTP-induced parkinsonism in primates. *J. Neural Transm. Park. Dis. Dement. Sect.* 1991; 3:63–72. [PubMed: 2064731]
- Boireau A, Dubedat P, Laduron PM, Doble A, Blanchard JC. Preferential decrease in dopamine utilization in prefrontal cortex by zopiclone, diazepam and zolpidem in unstressed rats. *J. Pharm. Pharmacol.* 1990; 42:562–565. [PubMed: 1981584]
- Briggs JP. The zebrafish: a new model organism for integrative physiology. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2002; 282:R3–R9. [PubMed: 11742817]
- Buske C, Gerlai R. Early embryonic ethanol exposure impairs shoaling and the dopaminergic and serotonergic systems in adult zebrafish. *Neurotoxicol. Teratol.* 2011; 33:698–707. [PubMed: 21658445]
- Cao X, Li XM, Mousseau DD. Calcium alters monoamine oxidase-A parameters in human cerebellar and rat glial C6 cell extracts: possible influence by distinct signalling pathways. *Life Sci.* 2009; 85:262–268. [PubMed: 19539632]
- Castorina M, Ferraris L. Acetyl-L-carnitine affects aged brain receptorial system in rodents. *Life Sci.* 1994; 54:1205–1214. [PubMed: 8164502]
- Catena-Dell'Oso M, Fagiolini A, Rotella F, Baroni S, Marazziti D. Glutamate system as target for development of novel antidepressants. *CNS Spectr.* 2013; 18:188–198. [PubMed: 23369807]
- Chang JW, Lee WY, Milstien S, Kang UJ. A site-specific mutation of tyrosine hydroxylase reduces feedback inhibition by dopamine in genetically modified cells grafted in parkinsonian rats. *J. Neurochem.* 2002; 83:141–149. [PubMed: 12358737]
- Chatterjee M, Verma R, Ganguly S, Palit G. Neurochemical and molecular characterization of ketamine-induced experimental psychosis model in mice. *Neuropharmacology*. 2012; 63:1161–1171. [PubMed: 22683513]
- Corssen G, Miyasaka M, Domino EF. Changing concepts in pain control during surgery: dissociative anesthesia with CI-581. A progress report. *Anesth. Analg.* 1968; 47:746–759. [PubMed: 5749338]
- Cuevas E, Trickler WJ, Guo X, Ali SF, Paule MG, Kanungo J. Acetyl L-carnitine protects motor neurons and Rohon-Beard sensory neurons against ketamine-induced neurotoxicity in zebrafish embryos. *Neurotoxicol. Teratol.* 2013; 39:69–76. [PubMed: 23896048]

- da Silva RB, Siebel AM, Bonan CD. The role of purinergic and dopaminergic systems on MK-801-induced antidepressant effects in zebrafish. *Pharmacol. Biochem. Behav.* 2015
- Daubner SC, Le T, Wang S. Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch. Biochem. Biophys.* 2011; 508:1–12. [PubMed: 21176768]
- Domino EF, Zsigmond EK, Domino LE, Domino KE, Kothary SP, Domino SE. Plasma levels of ketamine and two of its metabolites in surgical patients using a gas chromatographic mass fragmentographic assay. *Anesth. Analg.* 1982; 61:87–92. [PubMed: 7198883]
- Doskeland AP, Flatmark T. Ubiquitination of soluble and membrane-bound tyrosine hydroxylase and degradation of the soluble form. *Eur. J. Biochem.* 2002; 269:1561–1569. [PubMed: 11874472]
- Egashira T, Sakai K, Sakurai M, Takayama F. Calcium disodium edetate enhances type A monoamine oxidase activity in monkey brain. *Biol. Trace Elem. Res.* 2003; 94:203–211. [PubMed: 12972688]
- Eisenhofer G, Aneman A, Friberg P, Hooper D, Fandriks L, Lonroth H, Hunyady B, Mezey E. Substantial production of dopamine in the human gastrointestinal tract. *J. Clin. Endocrinol. Metab.* 1997; 82:3864–3871. [PubMed: 9360553]
- Fariello RG, Ferraro TN, Golden GT, DeMattei M. Systemic acetyl-L-carnitine elevates nigral levels of glutathione and GABA. *Life Sci.* 1988; 43:289–292. [PubMed: 3398700]
- Felix LM, Antunes LM, Coimbra AM. Ketamine NMDA receptor-independent toxicity during zebrafish (*Danio rerio*) embryonic development. *Neurotoxicol. Teratol.* 2014; 41:27–34. [PubMed: 24287188]
- Fitzgerald JC, Ufer C, Billett EE. A link between monoamine oxidase-A and apoptosis in serum deprived human SH-SY5Y neuroblastoma cells. *J. Neural. Transm. (Vienna).* 2007; 114:807–810. [PubMed: 17393061]
- Geisslinger G, Hering W, Thomann P, Knoll R, Kamp HD, Brune K. Pharmacokinetics and pharmacodynamics of ketamine enantiomers in surgical patients using a stereoselective analytical method. *Br. J. Anaesth.* 1993; 70:666–671. [PubMed: 8329260]
- Haberny KA, Paule MG, Scallet AC, Sistare FD, Lester DS, Hanig JP, Slikker W Jr. Ontogeny of the N-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. *Toxicol. Sci.* 2002; 68:9–17. [PubMed: 12075105]
- Hamilton WR, Trickler WJ, Robinson BL, Paule MG, Ali SF. Effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on retinal dopaminergic system in mice. *Neurosci. Lett.* 2012; 515:107–110. [PubMed: 22414866]
- Harsing LG Jr, Sershen H, Toth E, Hashim A, Ramacci MT, Lajtha A. Acetyl-L-carnitine releases dopamine in rat corpus striatum: an in vivo microdialysis study. *Eur. J. Pharmacol.* 1992; 218:117–121. [PubMed: 1397026]
- Idvall J, Ahlgren I, Aronsen KR, Stenberg P. Ketamine infusions: pharmacokinetics and clinical effects. *Br. J. Anaesth.* 1979; 51:1167–1173. [PubMed: 526385]
- Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vockler J, Dikranian K, Tenkova TI, Stefovskaya V, Turski L, Olney JW. Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science.* 1999; 283:70–74. [PubMed: 9872743]
- Irifune M, Shimizu T, Nomoto M. Ketamine-induced hyperlocomotion associated with alteration of presynaptic components of dopamine neurons in the nucleus accumbens of mice. *Pharmacol. Biochem. Behav.* 1991; 40:399–407. [PubMed: 1805243]
- Irifune M, Fukuda T, Nomoto M, Sato T, Kamata Y, Nishikawa T, Mietani W, Yokoyama K, Sugiyama K, Kawahara M. Effects of ketamine on dopamine metabolism during anesthesia in discrete brain regions in mice: comparison with the effects during the recovery and subanesthetic phases. *Brain Res.* 1997; 763:281–284. [PubMed: 9296573]
- Kanungo J, Cuevas E, Ali SF, Paule MG. L-Carnitine rescues ketamine-induced attenuated heart rate and MAPK (ERK) activity in zebrafish embryos. *Reprod. Toxicol.* 2012; 33:205–212. [PubMed: 22027688]
- Kanungo J, Cuevas E, Ali SF, Paule MG. Ketamine induces motor neuron toxicity and alters neurogenic and proneural gene expression in zebrafish. *J. Appl. Toxicol.* 2013; 33:410–417. [PubMed: 22045596]
- Kanungo J, Cuevas E, Ali SF, Paule MG. Zebrafish model in drug safety assessment. *Curr. Pharm. Des.* 2014; 20:5416–5429. [PubMed: 24502596]

- Kapur S, Seeman P. Ketamine has equal affinity for NMDA receptors and the high-affinity state of the dopamine D2 receptor. *Biol. Psychiatry*. 2001; 49:954–957. [PubMed: 11398792]
- Kelland MD, Chiodo LA, Freeman AS. Anesthetic influences on the basal activity and pharmacological responsiveness of nigrostriatal dopamine neurons. *Synapse*. 1990; 6:207–209. [PubMed: 1978421]
- Key B, Devine CA. Zebrafish as an experimental model: strategies for developmental and molecular neurobiology studies. *Methods Cell Sci*. 2003; 25:1–6. [PubMed: 14739581]
- Kohrs R, Durieux ME. Ketamine: teaching an old drug new tricks. *Anesth. Analg*. 1998; 87:1186–1193. [PubMed: 9806706]
- Kosenko EA, Venediktova NI, Kaminskii Iu G. Calcium and ammonia stimulate monoamine oxidase A activity in brain mitochondria. *Izv. Akad. Nauk Ser. Biol*. 2003:542–546.
- Koshikawa N, Tomiyama K, Omiya K, Kobayashi M. Ketamine anaesthesia has no effect on striatal dopamine metabolism in rats. *Brain Res*. 1988; 444:394–396. [PubMed: 3359305]
- Kuhn DM, Aretha CW, Geddes TJ. Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. *J. Neurosci*. 1999; 19:10289–10294. [PubMed: 10575026]
- Kumer SC, Vrana KE. Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem*. 1996; 67:443–462. [PubMed: 8764568]
- Kung TS, Richardson JR, Cooper KR, White LA. Developmental deltamethrin exposure causes persistent changes in dopaminergic gene expression, neurochemistry, and locomotor activity in zebrafish. *Toxicol. Sci*. 2015
- Lahti AC, Weiler MA, Tamara Michaelidis BA, Parwani A, Tamminga CA. Effects of ketamine in normal and schizophrenic volunteers. *Neuropsychopharmacology*. 2001; 25:455–467. [PubMed: 11557159]
- Lantz-McPeak S, Guo X, Cuevas E, Dumas M, Newport GD, Ali SF, Paule MG, Kanungo J. Developmental toxicity assay using high content screening of zebrafish embryos. *J. Appl. Toxicol*. 2015; 35:261–272. [PubMed: 24871937]
- Larsen B, Hoff G, Wilhelm W, Buchinger H, Wanner GA, Bauer M. Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood. *Anesthesiology*. 1998; 89:1218–1227. [PubMed: 9822011]
- Levin ED, Sledge D, Roach S, Petro A, Donerly S, Linney E. Persistent behavioral impairment caused by embryonic methylphenidate exposure in zebrafish. *Neurotoxicol. Teratol*. 2011; 33:668–673. [PubMed: 21741476]
- Li Y, Zhu ZR, Ou BC, Wang YQ, Tan ZB, Deng CM, Gao YY, Tang M, So JH, Mu YL, et al. Dopamine D2/D3 but not dopamine D1 receptors are involved in the rapid antidepressant-like effects of ketamine in the forced swim test. *Behav. Brain Res*. 2015; 279:100–105. [PubMed: 25449845]
- Liu F, Patterson TA, Sadovova N, Zhang X, Liu S, Zou X, Hanig JP, Paule MG, Slikker W Jr, Wang C. Ketamine-induced neuronal damage and altered N-methyl-D-aspartate receptor function in rat primary forebrain culture. *Toxicol. Sci*. 2013; 131:548–557. [PubMed: 23065140]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
- Malhotra AK, Pinals DA, Adler CM, Elman I, Clifton A, Pickar D, Breier A. Ketamine-induced exacerbation of psychotic symptoms and cognitive impairment in neuroleptic-free schizophrenics. *Neuropsychopharmacology*. 1997; 17:141–150. [PubMed: 9272481]
- Maxwell CR, Ehrlichman RS, Liang Y, Trief D, Kanes SJ, Karp J, Siegel SJ. Ketamine produces lasting disruptions in encoding of sensory stimuli. *J. Pharmacol. Exp. Ther*. 2006; 316:315–324. [PubMed: 16192313]
- McMillan CR, Sharma R, Ottenhof T, Niles LP. Modulation of tyrosine hydroxylase expression by melatonin in human SH-SY5Y neuroblastoma cells. *Neurosci. Lett*. 2007; 419:202–206. [PubMed: 17482356]
- Meiser J, Weindl D, Hiller K. Complexity of dopamine metabolism. *Cell. Commun. Signal*. 2013; 11:34. [PubMed: 23683503]

- Morgan CJ, Muetzelfeldt L, Curran HV. Consequences of chronic ketamine self-administration upon neurocognitive function and psychological wellbeing: a 1-year longitudinal study. *Addiction*. 2010; 105:121–133. [PubMed: 19919593]
- Narendran R, Frankle WG, Keefe R, Gil R, Martinez D, Slifstein M, Kegeles LS, Talbot PS, Huang Y, Hwang DR, et al. Altered prefrontal dopaminergic function in chronic recreational ketamine users. *Am. J. Psychiatry*. 2005; 162:2352–2359. [PubMed: 16330601]
- Niesters M, Khalili-Mahani N, Martini C, Aarts L, van Gerven J, van Buchem MA, Dahan A, Rombouts S. Effect of subanesthetic ketamine on intrinsic functional brain connectivity: a placebo-controlled functional magnetic resonance imaging study in healthy male volunteers. *Anesthesiology*. 2012; 117:868–877. [PubMed: 22890117]
- Owolabi AR, Akanmu MA, Ukponmwan OE. Functional reactivity of the dopaminergic system following acute and chronic ketamine treatments. *Naunyn Schmiedeberg's Arch. Pharmacol*. 2008; 378:117–124. [PubMed: 18408917]
- Palacios HH, Yendluri BB, Parvathaneni K, Shadlinski VB, Obrenovich ME, Leszek J, Gokhman D, Gasiorowski K, Bragin V, Aliev G. Mitochondrion-specific anti-oxidants as drug treatments for Alzheimer disease. *CNS Neurol. Disord. Drug Targets*. 2011; 10:149–162. [PubMed: 21222631]
- Parnig C, Seng WL, Semino C, McGrath P. Zebrafish: a preclinical model for drug screening. *Assay Drug Dev. Technol*. 2002; 1:41–48. [PubMed: 15090155]
- Paule MG, Li M, Allen RR, Liu F, Zou X, Hotchkiss C, Hanig JP, Patterson TA, Slikker W Jr, Wang C. Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. *Neurotoxicol. Teratol*. 2011; 33:220–230. [PubMed: 21241795]
- Pettegrew JW, Levine J, McClure RJ. Acetyl-l-carnitine physical-chemical, metabolic, and therapeutic properties: relevance for its mode of action in Alzheimer's disease and geriatric depression. *Mol. Psychiatry*. 2000; 5:616–632. [PubMed: 11126392]
- Redmond DE Jr, Baulu J, Murphy DL, Loriaux DL, Zeigler MG, Lake CR. The effects of testosterone on plasma and platelet monoamine oxidase (MAO) and plasma dopamine-beta-hydroxylase (DBH) activities in the male rhesus monkey. *Psychosom. Med*. 1976; 38:315–326. [PubMed: 824661]
- Ren G, Li S, Zhong H, Lin S. Zebrafish tyrosine hydroxylase 2 gene encodes tryptophan hydroxylase. *J. Biol. Chem*. 2013; 288:22451–22459. [PubMed: 23754283]
- Riehl R, Kyzar E, Allain A, Green J, Hook M, Monnig L, Rhymes K, Roth A, Pham M, Razavi R, et al. Behavioral and physiological effects of acute ketamine exposure in adult zebrafish. *Neurotoxicol. Teratol*. 2011; 33:658–667. [PubMed: 21683787]
- Robinson BL, Dumas M, Paule MG, Ali SF, Kanungo J. Opposing effects of ketamine and acetyl l-carnitine on the serotonergic system of zebrafish. *Neurosci. Lett*. 2015; 607:17–22. [PubMed: 26365406]
- Rowland LM. Subanesthetic ketamine: how it alters physiology and behavior in humans. *Aviat. Space Environ. Med*. 2005; 76:C52–C58. [PubMed: 16018330]
- Rujescu D, Bender A, Keck M, Hartmann AM, Ohl F, Raeder H, Giegling I, Genius J, McCarley RW, Moller HJ, et al. A pharmacological model for psychosis based on N-methyl-d-aspartate receptor hypofunction: molecular, cellular, functional and behavioral abnormalities. *Biol. Psychiatry*. 2006; 59:721–729. [PubMed: 16427029]
- Samantaray S, Chandra G, Mohanakumar KP. Calcium channel agonist, (+/-)-Bay K8644, causes a transient increase in striatal monoamine oxidase activity in Balb/c mice. *Neurosci. Lett*. 2003; 342:73–76. [PubMed: 12727321]
- Santarelli M, Granato A, Sbriccoli A, Gobbi G, Janiri L, Minciacchi D. Alterations of the thalamo-cortical system in rats prenatally exposed to ethanol are prevented by concurrent administration of acetyl-l-carnitine. *Brain Res*. 1995; 698:241–247. [PubMed: 8581491]
- Sershen H, Harsing LG Jr, Banay-Schwartz M, Hashim A, Ramacci MT, Lajtha A. Effect of acetyl-l-carnitine on the dopaminergic system in aging brain. *J. Neurosci. Res*. 1991; 30:555–559. [PubMed: 1839317]
- Slikker W Jr, Zou X, Hotchkiss CE, Divine RL, Sadovova N, Twaddle NC, Doerge DR, Scallet AC, Patterson TA, Hanig JP, et al. Ketamine-induced neuronal cell death in the perinatal rhesus monkey. *Toxicol. Sci*. 2007; 98:145–158. [PubMed: 17426105]

- Snyder JW, Kyle ME, Ferraro TN. l-Carnitine delays the killing of cultured hepatocytes by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Arch. Biochem. Biophys.* 1990; 276:132–138. [PubMed: 2297220]
- Stefani MR, Moghaddam B. Transient N-methyl-d-aspartate receptor blockade in early development causes lasting cognitive deficits relevant to schizophrenia. *Biol. Psychiatry.* 2005; 57:433–436. [PubMed: 15705361]
- Steffen V, Santiago M, de la Cruz CP, Revilla E, Machado A, Cano J. Effect of intraventricular injection of 1-methyl-4-phenylpyridinium: protection by acetyl-l-carnitine. *Hum. Exp. Toxicol.* 1995; 14:865–871. [PubMed: 8588946]
- Suen MF, Chan WS, Hung KW, Chen YF, Mo ZX, Yung KK. Assessments of the effects of nicotine and ketamine using tyrosine hydroxylase-green fluorescent protein transgenic zebrafish as biosensors. *Biosens. Bioelectron.* 2013; 42:177–185. [PubMed: 23202349]
- Svensson A, Carlsson ML, Carlsson A. Crucial role of the accumbens nucleus in the neurotransmitter interactions regulating motor control in mice. *J. Neural Transm. Gen. Sect.* 1995; 101:127–148. [PubMed: 8695043]
- Tan S, Lam WP, Wai MS, Yu WH, Yew DT. Chronic ketamine administration modulates midbrain dopamine system in mice. *PLoS One.* 2012; 7:e43947. [PubMed: 22937133]
- Tolu P, Masi F, Leggio B, Scheggi S, Tagliamonte A, De Montis MG, Gambarana C. Effects of long-term acetyl-l-carnitine administration in rats: I. increased dopamine output in mesocorticolimbic areas and protection toward acute stress exposure. *Neuropsychopharmacology.* 2002; 27:410–420. [PubMed: 12225698]
- Trickler WJ, Guo X, Cuevas E, Ali SF, Paule MG, Kanungo J. Ketamine attenuates cytochrome p450 aromatase gene expression and estradiol-17beta levels in zebrafish early life stages. *J. Appl. Toxicol.* 2014; 34:480–488. [PubMed: 23696345]
- Virmani A, Gaetani F, Imam S, Binienda Z, Ali S. Possible mechanism for the neuroprotective effects of l-carnitine on methamphetamine-evoked neurotoxicity. *Ann. N. Y. Acad. Sci.* 2003; 993:197–207. (discussion 287-8). [PubMed: 12853314]
- Wang C, Sadovova N, Hotchkiss C, Fu X, Scallet AC, Patterson TA, Hanig J, Paule MG, Slikker W Jr. Blockade of N-methyl-d-aspartate receptors by ketamine produces loss of postnatal day 3 monkey frontal cortical neurons in culture. *Toxicol. Sci.* 2006; 91:192–201. [PubMed: 16500925]
- Wong WK, Ou XM, Chen K, Shih JC. Activation of human monoamine oxidase B gene expression by a protein kinase C MAPK signal transduction pathway involves c-Jun and Egr-1. *J. Biol. Chem.* 2002; 277:22222–22230. [PubMed: 11956220]
- Ylitalo P, Saarnivaara L, Ahtee L. Effect of ketamine anaesthesia on the content of monoamines and their metabolites in the rat brain. *Acta Anaesthesiol. Scand.* 1976; 20:216–220. [PubMed: 961330]
- Yu H, Li Q, Wang D, Shi L, Lu G, Sun L, Wang L, Zhu W, Mak YT, Wong N, et al. Mapping the central effects of chronic ketamine administration in an adolescent primate model by functional magnetic resonance imaging (fMRI). *Neurotoxicology.* 2012; 33:70–77. [PubMed: 22178134]
- Zhang J, Chiodo LA, Freeman AS. Effects of phencyclidine, MK-801 and 1,3-di(2-tolyl)guanidine on non-dopaminergic midbrain neurons. *Eur. J. Pharmacol.* 1993; 230:371–374. [PubMed: 8440313]
- Zou X, Sadovova N, Patterson TA, Divine RL, Hotchkiss CE, Ali SF, Hanig JP, Paule MG, Slikker W Jr, Wang C. The effects of l-carnitine on the combination of, inhalation anesthetic-induced developmental, neuronal apoptosis in the rat frontal cortex. *Neuroscience.* 2008; 151:1053–1065. [PubMed: 18201836]

Fig. 1. Effects of low doses of ketamine on DA, DOPAC and HVA levels in zebrafish embryos. Embryos at 52 hpf were treated with 0.1–0.3 mM ketamine. Twenty four hours post exposure (76 hpf) embryos were processed for DA (A) as well as its metabolites, DOPAC (B) and HVA (C) measurements using HPLC/EC. Values are expressed in ng/μg protein in embryo extracts (means $\pm$ SDs). One-way ANOVA with post-hoc analyses were used to assess statistical significance in variation in DA, DOPAC and HVA levels between the experimental groups as well as with controls.

Fig. 2.

Effects of ketamine at a dose relevant to the human anesthetic dose and ALCAR on DA, DOPAC and HVA levels in zebrafish embryos. Embryos at 52 hpf were treated with 2 mM ketamine, 2 mM ketamine plus 0.5 mM ALCAR or 0.5 mM ALCAR alone. Twenty four hours post exposure (76 hpf), embryos were processed for DA (A) as well as its metabolites, DOPAC (B) and HVA (C) measurements using HPLC/EC. Values are expressed in ng/μg protein in the embryo extracts as means $\pm$ SDs. One-way ANOVA with post-hoc analyses were used to assess statistical significance (* P = <0.05; ** P = <0.001; *** P = <0.0001) of

variations in levels of DA and its metabolites, DOPAC and HVA between the experimental groups as well as with controls.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 3.

Effects of ketamine and ALCAR on DA metabolism in zebrafish embryos. Embryos at 52 hpf were treated with lower doses of ketamine (0.1–0.3 mM; upper panels) or 2 mM ketamine, 2 mM ketamine plus 0.5 mM ALCAR or 0.5 mM ALCAR alone (lower panels). Twenty hours post exposure (76 hpf) embryos were processed for DA as well as its metabolites, DOPAC and HVA measurements using HPLC/EC. The rate of DA metabolism was modeled using the metabolite/DA ratios. The ratios for the embryos treated with 0.1–0.3mM ketamine are presented in the top panels, (A) DOPAC/DA and (C) HVA/DA. The ratios for the embryos treated with 2 mM ketamine, 2 mM ketamine plus 0.5 mM ALCAR or 0.5 mM ALCAR alone are presented in the lower panels, (B) DOPAC/DA and (D) HVA/DA. Values are expressed as means $\pm$ SDs. One-way ANOVA with post-hoc analyses were used to assess statistical significance (* P = <0.05; ** P = <0.001; *** P = <0.0001) of the variation in values between the experimental groups as well as with the controls.

Fig. 4.

Effects of ketamine and ALCAR on TH gene expression. Embryos at 52 hpf were treated with lower doses of ketamine (0.1–0.3 mM) (A) as well as 2 mM ketamine, 2 mM ketamine plus 0.5 mM ALCAR or 0.5 mM ALCAR (B) alone for 24 h. Total RNA was extracted from the embryos (76 hpf) and after first strand cDNA synthesis from the RNA, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. The 2^{-CT} method was used to determine relative gene expression for TH. The GAPDH gene served as the internal control for all qRT-PCR experiments. Data were averaged and shown as normalized (fold-

change over control) relative gene expression $\pm$ SDs. One-way ANOVA with comparison post-hocs were used to determine statistical significance with $P = <0.05$ (*).

Fig. 5.

Effects of ketamine and ALCAR on MAO and COMT gene expression. The schematic diagram shows dopamine (DA), metabolized to DOPAC, 3-MT (3-Methoxytyramine) and HVA, by two enzymes, MAO and COMT (A). Embryos at 52 hpf were treated with lower doses of ketamine (0.1–0.3 mM) (B) as well as 2 mM ketamine, 2 mM ketamine plus 0.5 mM ALCAR or 0.5 mM ALCAR (C) alone for 24 h. Total RNA was extracted from the embryos (76 hpf) and after first strand cDNA synthesis from the RNA, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. The 2^{-CT} method was used to

determine relative gene expression for MAO and COMT. The GAPDH gene served as the internal control for all qRT-PCR experiments. Data were averaged and shown as normalized (fold-change over control) relative gene expression $\pm$ SDs. One-way ANOVA with comparison post-hocs were used to determine statistical significance with $P = <0.05$ (*).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript