HHS Public Access

Author manuscript

J Appl Toxicol. Author manuscript; available in PMC 2017 June 19.

Published in final edited form as:

J Appl Toxicol. 2014 May ; 34(5): 480–488. doi:10.1002/jat.2888.

Ketamine attenuates cytochrome p450 aromatase gene expression and estradiol-17β levels in zebrafish early life stages

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Abstract

Ketamine, a dissociative anesthetic, is a noncompetitive antagonist of N-methyl-D-aspartate-type glutamate receptors. In rodents and non-human primates as well as in zebrafish embryos, ketamine has been shown to be neurotoxic. In cyclic female rats, ketamine has been shown to decrease serum estradiol-17β(E2) levels. E2 plays critical roles in neurodevelopment and neuroprotection. Cytochrome p450 (CYP) aromatase catalyzes E2 synthesis from androgens. Although ketamine down-regulates a number of CYP enzymes in rodents, its effect on the CYP aromatase (CYP19) is not known. Zebrafish have been used as a model system for examining mechanisms underlying drug effects. Here, using wild-type (WT) zebrafish (*Danio rerio*) embryos, we demonstrate that ketamine significantly reduced E2 levels compared with the control. However, the testosterone level was elevated in ketamine-treated embryos. These results are concordant with data from mammalian studies. Ketamine also attenuated the expression of the ovary form of CYP aromatase (*cyp19a1a*) at the transcriptional level but not the brain form of aromatase, *cyp19a1b*. Exogenous E2 potently induced the expression of *cyp19a1b* and *vtg 1*, both validated biomarkers of estrogenicity and endocrine disruption, but not *cyp19a1a* expression. Attenuation of activated ERK/MAPK levels, reportedly responsible for reduced human *cyp19* transcription, was also observed in ketamine-treated embryos. These results suggest that reduced E2 levels in ketamine-treated embryos may have resulted from the suppression of *cyp19a1a* transcription. Published 2013. This article is a U.S. Government work and is in the public domain in the USA.

Keywords

ketamine; zebrafish; estradiol-17β; CYP aromatase; gene expression; testosterone

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Introduction

Ketamine [2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone], a pediatric anesthetic, is thought to act primarily through blockade of N-methyl-D-aspartate (NMDA)-type glutamate receptors to provide analgesia/anesthesia to children for painful procedures (Kohrs and Durieux, 1998). Several previous 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 (Lahti *et al.*, 2001; Larsen *et al.*, 1998; Malhotra *et al.*, 1997; Maxwell *et al.*, 2006) and primates (Haberny *et al.*, 2002; Slikker et al., 2007; Wang *et al.*, 2006) and these effects can manifest as later disruptions in cognitive function (Paule *et al.*, 2011). We have previously reported that ketamine induces motor neuron toxicity in zebrafish embryos (Kanungo *et al.*, 2013).

Estrogens are known to play key roles in a wide range of physiological functions, including immune responses, the central nervous system and normal somatic cell growth (Filby and Tyler 2005; Gustafsson 2003). Among the anesthetics, ketamine, but not pentobarbital, has been shown to reduce serum concentrations of estradiol-17β (E2) in adult cyclic female rats (Lee *et al.*, 2000) although any direct action of anesthetics on the ovary is not known. Ketamine does not affect ovulation and serum levels of FSH (follicle-stimulating hormone) and LH (luteinizing hormone) in cyclic rats (Clarke and Doughton, 1983). E2 synthesis from androgens in gonadal and extragonadal tissues is carried out by cytochrome p450 (CYP) aromatase encoded by the gene *cyp19* (Meinhardt and Mullis, 2002). CYPs are a diverse superfamily of the heme-containing monooxygenase enzymes (Nebert and Dalton 2006; Nebert *et al.*, 1991) involved in the metabolism and bioactivation of both endogenous and exogenous chemicals (Nebert *et al.*, 1991; Zhang and Yang, 2009).

Zebrafish have a total of 94 CYP genes, distributed among 18 gene families found also in mammals (Goldstone *et al.*, 2010). While expressions of CYPs are transcriptionally regulated by a variety of xenobiotics (Gotoh, 2012), it has been shown that ketamine suppresses CYP3A4 gene expression in mammals (Chang *et al.*, 2009; Chen and Chen, 2010) that could result in endoplasmic reticulum stress, heat shock and apoptotic response (Acharya *et al.*, 2009). However, ketamine's effect on CYP19 aromatase is not known.

With its small size, prolific reproductive capacity and easy maintenance, 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). Exposure to environmental estrogens in oviparous organisms, such as zebrafish, induces a molecular response that is mediated by the estrogen receptor (ER) where binding leads to induction of more ER, increased production of estrogen and subsequent synthesis of the egg yolk precursor protein vitellogenin (vtg) in the liver (Jobling *et al.*, 2006). Induction of vtg is a widely accepted biomarker of exposure to estrogenic compounds (Sumpter and Jobling, 1995). C*yp19a1b* is the brain form of aromatase in zebrafish (Chiang *et al.*, 2001; Tchoudakova *et al.*, 2001) and is highly responsive to estrogens (Menuet *et al.*, 2005). Both the ER and aromatases expressed early in development in zebrafish and xenobiotic response elements have been shown to exist in the promoter region of *cyp19a1b*, thus, providing

another mechanistic link between these systems (Kazeto *et al.*, 2004). Data obtained from studies using a transgenic *cyp19a1*b-GFP zebrafish line indicate that *cyp19a1b* expression increases between 24 and 48 h post fertilization (hpf) whereas the expression is blocked in embryos exposed to anti-estrogens (Mouriec *et al.*, 2009). As the aromatase is highly expressed in radial glial cells that are progenitors for generating neurons throughout the zebrafish life cycle (Adolf *et al.*, 2006; Pellegrini *et al.*, 2005), estrogens are now strongly implicated in the process of embryonic, adult or reparative neurogenesis (Barha *et al.*, 2009; De Nicola *et al.*, 2009; Galea 2008; Garcia-Segura *et al.*, 2001; Martinez-Cerdeno *et al.*, 2006).

Previously, we have shown that ketamine induces developmental neurotoxicity in zebrafish embryos (Kanungo *et al.*, 2013). The goal of the present study was to evaluate whether in these embryos, ketamine modulated steroid hormone levels and expression of aromatase genes, *cyp19a1a* and *cyp19a1b*.

Materials and Methods

Materials

Ketamine (ketamine HCL) and E2 were purchased from Phoenix (St. Joseph, MO, USA) and Sigma (St. Louis, MO, USA), respectively. All high-performance liquid chromatography (HPLC) reagents, unless otherwise indicated, were obtained from Sigma (St. Louis, MO, USA).

Animals

Adult wild-type (WT) 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 08.30 hours. 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 08.30 hours. 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 of 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 further experiments.

Treatment of Zebrafish Embryos with Ketamine and E2

Embryos were developmentally staged according to methods described previously (Kimmel *et al.*, 1995). For ketamine exposure, ketamine (2.0 mM) was added directly to the embryo medium. E2 was used at a 1-nM concentration. Exposure was for 24 h in 6-well cell culture plates (20 embryos per well).

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 × 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 alkalinized with 0.35 ml of 0.2 mol l^{-1} borate buffer and sonicated at 30% intensity for 15 s and then extracted with dichloromethane: ethyl acetate (80:20 v/v) by mixing (75 × g, 10 min). The samples were centrifuged (1500 *g*, 3 min) and the organic layer was transferred to a borate test, 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 µl was injected into the column, and the amount of ketamine was determined by an HPLC method described below.

The amount of ketamine was determined by 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 × 4.6 mm, 5 µm) (Agilent, Santa Clara, CA, USA) with a simple mobile phase consisting of acetonitrile: 0.03 mol l^{-1} phosphate buffer (23:77% v/v) adjusted to pH 7.2 at a flow rate of 1.5 ml min^{-1}. 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 µmol l^{-1}). 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 per embryo) ± SD.

Determination of E2 Levels in Zebrafish Embryos by Reverse Phase HPLC

The embryos were collected in microcentrifuge tubes (1.5 ml), centrifuged in a microcentrifuge (2000 rpm) 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 alkalinized with 0.35 ml of perchloric acid (0.2 N) and sonicated at 30% intensity for 15 s and then centrifuged (14000 *g*, 20 min) and the aqueous layer was transferred to a borate test tube and evaporated to dryness at 45 °C. The dried residue was reconstituted in 0.1 ml of mobile phase and 50 µl was injected into the column, and the amount of E2 was determined by an HPLC method described below.

The amount of E2 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 E2 was achieved on a SB-C18 Zorbax column (150 × 4.6 mm, 5 um) (Agilent) with a simple mobile phase consisting of acetonitrile: water (35:65% v/v) at a flow rate of 1.5 ml min^{-1}. The effluents were monitored at 215 nm and quantified using the area under the peak from standard solutions dissolved in mobile phase (0.15 to 50 µmol l^{-1}). The data are expressed as mean (amount/embryo) ± SD from triplicate samples.

RNA Extraction and cDNA Synthesis

Total RNA (from 50 pooled embryos/treatment group) was extracted from whole embryos using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). An aliquot of each RNA sample was used to spectrophotometrically (using a NanoDrop ND-1000; NanoDrop Technology, Wilmington, DE, USA) determine RNA quality (A260/A280 > 2.0) and concentration. First-strand cDNA was synthesized from total RNA (1 μg; 20 μl final reaction volume) with oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Primers

Zebrafish gene-specific primers (Table 1) were used for the quantitative real-time polymerase chain reaction (RT-qPCR) assays to quantify GAPDH, *cyp19a1a* (GenBank entry no. AF226620), *cyp19a1b* (GenBank entry no. AF226619) and *vtg1* (GenBank entry no. AF406784.1).

RT-qPCR

Real-time qPCR 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 $2^{-\Delta\Delta Ct}$ method was used to determine the relative gene expression (Livak and Schmittgen, 2001). The GAPDH gene was the internal control for all qPCR experiments. Data from each group ($n = 3$) were averaged and shown as normalized gene expression ±SD. One-way ANOVA and Holm–Sidak pair-wise multiple comparison post-hocs (Sigma Stat 3.1 for analysis) were used to determine statistical significance with $P < 0.05$.

Sample Preparation for ELISA to Determine Testosterone and E2 Levels in Embryos

The embryos (125 per sample in triplicate) at 48 hpf were treated either with or without ketamine (2 mM) for 24 h. Post-treatment, embryos were collected in microcentrifuge tubes (1.5 ml). The embryos were suspended in deionized water (0.5 ml), sonicated (30% intensity for 30 s) and then centrifuged (14000 *g*, 20 min). The aqueous layer was transferred to a glass vial and dichloromethane (2 ml) was added followed by thorough mixing. The mixture was incubated on a shaker for 2 h. After incubation, the samples were centrifuged (1000 *g*, 30 s.), and the bottom organic layers was transferred to a clean test tube and evaporated to dryness at 45 °C overnight. The dried residue was reconstituted in 0.4 ml of EIA buffer supplied with the ELISA kits, and the amount of E2 or testosterone was determined by ELISA methods as described in the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA). The data are presented as the amount of compound of interest per mg protein as determined by the Pierce BCA protein assay.

Electrophoresis and Western Blot Analysis

Embryos were prepared for immunoblotting (Western blot) through sonication (one embryo per sample) in Eppendorf tubes in loading buffer [0.125 M Tris–HCl, pH 6.8; 4% sodium dodecyl sulfate (SDS); 20% glycerol; 0.2 M dithiothreitol (DTT); 0.02% bromophenol blue in distilled water) along with an additional 0.1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (protease inhibitor)]. Protein samples (one embryo equivalent/lane) were run in 4–20% gradient SDS-polyacrylamide gels purchased from Bio-Rad (Hercules, CA, USA) at 30–32 mA for protein separation. Proteins were then transferred onto a nitrocellulose membrane in transfer buffer (20% methanol, 25 mM Tris, 192 mM glycine, 0.1% SDS in distilled water). Transfer membranes were blocked with 2% bovine serum albumin (BSA) in Tris-borate saline with 0.5% Tween 20 (blocking buffer) and probed with phospho-MAPK specific antimouse IgG diluted in blocking buffer (1:2500; Sigma-Aldrich, St. Louis, MO, USA). After development for chemiluminescent signals, the same membrane was stripped from the bound antibodies using the stripping buffer (Thermo Scientific, Rockford, IL, USA) and reprobed with the total ERK1 anti-rabbit IgG (1:500; Santa Cruz Biotech., Santa Cruz, CA, USA). Advanced ECL Western Blotting Substrate (GE Healthcare, Piscataway, NJ, USA) was used to detect HRP-conjugate bound secondary antibodies. For quantification, densitometry of the immunoblot signals was performed using ImageJ (National Institutes of Health, Bethesda, MD).

Results

Time Course of Changes in Ketamine Accumulation in Zebrafish Embryos

Based on the reports that ketamine decreases plasma levels of E2 in rodents, and our previous studies showing ketamine as an inducer of neurotoxicity in zebrafish embryos (Kanungo *et al.*, 2013), we tested whether similar effects of ketamine in reducing E2 also manifest in zebrafish embryos, a suitable vertebrate animal model for drug screening and drug safety assessments. To this end, WT zebrafish embryos at 48 hpf were treated with 2 mM ketamine for up to 24 h (static exposure), the dose and duration selected on the basis of our previous report on ketamine-induced neurotoxicity (Kanungo *et al.*, 2013). Internal ketamine concentrations in these embryos were determined by reverse-phase HPLC and were well within the limits of quantification at 2, 4 and 20 h of static exposures. At a ketamine dose 2 mM, the amount of ketamine absorbed by the embryos was determined to be ~7.27 μM per embryo (Fig. 1). Differences in the amount of ketamine absorbed by the embryos after 2, 4 and 20 h were minimal and insignificant, indicating that the internal concentrations of ketamine reached steady-state conditions in the embryos within 2 h of exposure.

Reduced E2 and Elevated Testosterone Levels in Ketamine-Treated Embryos

Ketamine-treated embryos were processed to determine the internal levels of E2 using reverse-phase HPLC and the observed amounts fell well within the limits of quantification. The results showed a significant reduction in E2 levels (approximately four-fold) in ketamine-treated embryos compared with untreated controls (Fig. 2). The four-fold decrease in E2 suggested that ketamine may have interfered with E2 synthesis that is mediated by cyp19 aromatase, otherwise known as estrogen synthase. It is important to note that without

sex chromosomes, zebrafish early life stages up to 21 days post-fertilization (dpf) develop as females and sex differentiation begins at 21 dpf.

Further analysis using ELISA demonstrated a significant reduction in E2 levels but elevated testosterone levels in ketamine-treated embryos compared with the controls (Fig. 3).

Effects of E2 on the Expression of Estrogen-Responsive and Non-Responsive Genes

In order to test whether ketamine also altered the expression of cyp19 aromatases, we monitored the expression of both *cyp19a1a* and *cyp19a1b* aromatase genes along with their responsiveness to exogenous E2. First, as well-established controls, after RT-qPCR analyzes of cDNA derived from RNA isolated from 48 hpf embryos treated with E2 for 24 h (actual age of the embryo being 72 hpf during analysis), expression of both the aromatases and *vtg 1*, the gene encoding the estrogen-responsive, liver-synthesized protein vitellogenin, were quantified. The brain form of aromatase, *cyp19a1b*, and *vtg 1* were significantly induced by 1 nM E2, the lowest dose that reportedly evokes a response by estrogen-inducible genes. These genes serve as biomarkers of estrogenicity or endocrine disruption. However, E2 did not alter *cyp19a1a* expression (Fig. 4A) as it is known to be recalcitrant to E2 induction (Cheshenko *et al.*, 2007; McElroy *et al.*, 2012).

Ketamine's Effect on the Expression of CYP19 Aromatase Genes

The next step was to ascertain whether ketamine has any effect on the aromatase and *vtg 1* gene expression, embryos at 48 hpf were treated with 2 mM ketamine for 24 h (actual age at the time of analysis being 72 hpf). RT-qPCR analyzes showed that compared with the control, there was no significant difference in the expression of *vtg 1* or *cyp19a1b* transcripts (Fig. 4B). However, there was a significant reduction (~ 12-fold) in *cyp19a1a* expression. These results demonstrated that ketamine does not possess estrogenic activity. While the expression of brain aromatase, *cyp19a1b*, remained unaltered, suppression of the expression of ovary aromatase, *cyp19a1a*, by ketamine suggests that the significant reduction in E2 levels in the embryos could be the consequence of reduced expression of the latter, although both the aromatases are capable of mediating E2 synthesis from androgens.

Involvement of ERK/MAPK signaling in inducing *cyp19* transcription in a human cell line (MCF-7) have been reported (Catalano *et al.*, 2003; Ye *et al.*, 2009) and our previous study has shown that ketamine attenuates activated (phosphorylated) ERK/MAPK (p-ERK) levels in 28 hpf zebrafish embryos treated for 20 h with 2 mM ketamine (Kanungo *et al.*, 2012). Here, we analyzed the ERK/MAPK levels in the 48 hpf embryos treated for 24 h with 2 mM ketamine and the results showed a significant reduction in p-ERK levels although E2 did not induce any significant change (Fig. 5). These results suggested that similar to human MCF-7 cells, ERK/MAPK signaling may be involved in *cyp19a1a* transcription.

Discussion

Anesthetics have complex neuroendocrine effects and ketamine is known to block spontaneous GnRH (gonadotropin-releasing horomone) release and to decrease peripheral LH levels in adult rats (Cohen *et al.*, 1983; Emanuele *et al.*, 1987; Matzen *et al.*, 1987; Sherwood *et al.*, 1980). However, in male Sprague–Dawley rats, ketamine treatment

increased testosterone concentrations, but decreased LHRH (luteinizing hormone-releasing hormone) after 1 of administration and continued to significantly decrease after 24 h (Gould, 2008). In the present study, an effort to investigate whether ketamine influenced E2 concentration and CYP aromatase expression in zebrafish embryos was based on the reports that ketamine regulates a number of CYPs in mammals (Chen and Chen, 2010) and alters the serum concentrations of several hormones, such as progesterone, testosterone and E2 in cyclic rats (Lee *et al.*, 2000). Estrogens are fundamental in the growth and development of the ovary in females (Richards *et al.*, 1976), and spermatogenesis in males (Mahato *et al.*, 2001; O'Donnell *et al.*, 2001). In addition, estrogens are known to play key roles in the central nervous system, and normal somatic cell growth (Filby and Tyler, 2005; Gustafsson, 2003). It has been suggested that differences in estrogen levels in zebrafish embryos and larvae occur from an early developmental stage (Lee *et al.*, 2012). Our previous studies illustrated that 2 mM ketamine had an adverse effect on the central nervous system (Kanungo *et al.*, 2013). The present study shows the ketamine concentration to be ~ 7.27 μM per embryo after exposure to 2 mM ketamine for 24 h. The data are comparable with 10 μM ketamine inducing neuronal damage in primary neurons from rat forebrain in culture (Liu *et al.*, 2012). Additionally, our current data demonstrate that 48 hpf embryos with such low internal ketamine concentrations had significantly lower levels of E2 compared with the control, an effect concordant with that in mammals in which a single dose of ketamine reduces E2 levels (Lee *et al.*, 2000).

Ketamine-treated embryos showed an increase in testosterone level, which is in agreement with data reported in male boars (Estienne and Barb 2002), male collared peccary (*Tayassu tajacu*) (Hellgren *et al.*, 1985) and female Norway rats (Gould, 2008). In contrast, ketamine has been shown to cause a reduction in plasma testosterone levels in men (Oyama *et al.*, 1977), domestic tom cats (Johnstone and Bancroft, 1988) and cyclic female Sprague–Dawley rats (Lee *et al.*, 2000). In humans, although there have been some reports of decreased testosterone levels after general anesthesia, possibly owing to surgical trauma, more often an increase in the testosterone level occurred with ketamine anesthesia 1 h after surgery (Cartensen *et al.*, 1973). A lack of any significant change in testosterone levels in ketamine-treated male rhesus monkeys (Puri *et al.*, 1981; Zaidi *et al.*, 1982) and male cynomolgus (Malaivijitnond *et al.*, 1998) has also been reported. These differences may be attributed to variations in the dose and duration of ketamine treatment as well as sex and age of the subjects.

CYP19 aromatase is a crucial steroidogenic enzyme that catalyzes the final, rate-limiting step in the conversion of androgens into estrogens (Simpson *et al.*, 1994). In mammals, with the exception of pigs, there is a single CYP19 gene which is expressed in a variety of tissues (Simpson *et al.*, 2002). However, owing to genome duplication during evolution, teleosts including zebrafish contain a pair of aromatase genes: *cyp19a1a* and *cyp19a1b* (Cheshenko *et al.*, 2008). Changes in estradiol levels during sexual differentiation are directly correlated with the changes in aromatase transcript expression in the gonads of Japanese flounders (Kitano *et al.*, 1999). It has been reported that in zebrafish larvae exposed to E2 between 17 and 20 dpf, a time just prior to the onset of sex differentiation, there was no significant effect on *cyp19a1a* expression (Callard *et al.*, 2001). In contrast, short-term exposure of zebrafish larvae to E2 strongly up-regulated *cyp19a1b* expression (Cheshenko *et al.*, 2007; McElroy *et*

al., 2012) supporting similar reports on the response of zebrafish embryos and larvae exposed to E2 (Kishida and Callard, 2001). Therefore, *cyp19a1b* has proven to be a promising marker of estrogenic compounds in zebrafish early life stages (Menuet *et al.*, 2005). In contrast to the dramatic alteration of *cyp19a1b* expression, E2 has no effect on *cyp19a1a* expression in zebrafish larvae (Kazeto *et al.*, 2004; Trant *et al.*, 2001). Consistent with these reports, our data show 1 nM E2 significantly inducing *cyp19a1b* but not *cyp19a1a* expression in 48-hpf embryos.

Decades ago, in rodents, ketamine pretreatment was first reported to result in a two-fold increase in metabolism *in vitro* in hepatic microsomes suggesting a self-induction of ketamine metabolism (Marietta *et al.*, 1976) and implicating ketamine as an inducer of CYP enzymes. However, later studies in rodents showed that ketamine decreased CYP (CYP1A, CYP2A, CYP2B, CYP2C, CYP2D1 and CYP3A) activities (Loch *et al.*, 1995; Lupp *et al.*, 2003; Meneguz *et al.*, 1999). In human hepatoma HepG2 cells, ketamine suppresses CYP3A4 mRNA synthesis (Chang *et al.*, 2009). Moreover, ketamine-induced mitochondrial dysfunction and inhibition of calcium mobilization has been linked to suppression of CYP3A4 (Chang *et al.*, 2009). However, the exact molecular mechanism of ketamine-induced suppression of CYP gene expressions remains obscure. In our previous report, we have shown that ketamine-induced ERK/MAPK down regulation and an attenuated cardiac rate (Kanungo *et al.*, 2012). Suppression of cyp19 aromatase gene transcription in a human breast cancer cell line (MCF-7) has been shown to occur when ERK/MAPK activation is prevented (Catalano *et al.*, 2003; Ye *et al.*, 2009). Although our results are consistent in showing that ketamine caused attenuation of activated ERK/MAPK (p-ERK), whether ketamine's suppressive effect on *cyp19a1a* expression and concomitant reduction of E2 in zebrafish embryos are direct consequences of the modulated ERK/MAPK signaling pathway remain to be elucidated.

Aromatase knockout (ArKO) female mice are infertile as their reproductive organs do not develop properly (Simpson, 2004). These mice show impaired functionality in the amygdala and hypothalamus (Pierman *et al.*, 2008) and increased apoptosis in the frontal cortex (Hill *et al.*, 2008). Various studies on ArKO mice have implicated estrogen in neurodevelopment (Balthazart *et al.*, 1991; Sasahara *et al.*, 2007). Whether ketamine-induced down-regulation of the ovary form of aromatase (*cyp19a1a*) adversely affects the nervous system in zebrafish and is linked to ketamine-induced neurotoxicity (Kanungo *et al.*, 2013) need to be examined.

In conclusion, our data show that ketamine reduces E2 levels while elevating testosterone levels in zebrafish embryos. The single static exposure, however, did not alter estrogen-responsive gene (*vtg 1 and cyp19a1b*) transcription. Down-regulation of the ovary form of aromatase *cyp19a1a* further suggested that the reduced E2 levels in ketamine-treated embryos could result from the inhibition of its synthesis from androgens. Finally, our data showing ketamine's effects on E2 and testosterone levels in zebrafish embryos are concordant with data from mammals. These findings further emphasize the importance of cross-species comparisons to delineate molecular mode of action of drugs.

Acknowledgments

This work was supported by the National Center for Toxicological Research (NCTR)/U.S. Food and Drug Administration (FDA). We thank Melanie Dumas for zebrafish breeding.

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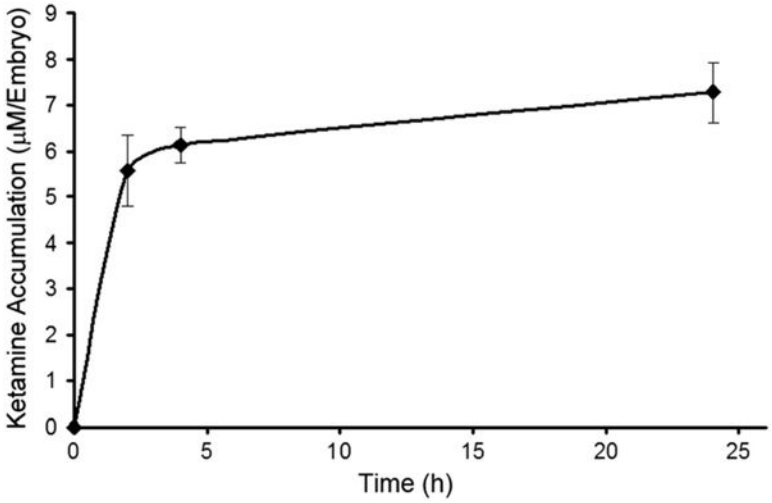

Figure 1.

Ketamine accumulation (internal ketamine concentrations) in zebrafish embryos after static exposure to 2 mM ketamine. Embryos at 48 h post fertilization (hpf) were exposed for 2, 4 and 24 h. Post exposure, embryos were washed extensively with quick changes of embryo water three times. Reverse-phase high-performance liquid chromatography (HPLC) was employed to measure ketamine concentrations in the embryos (n = 15 per group). Data from three separate estimations were averaged and are presented as the mean ± SD.

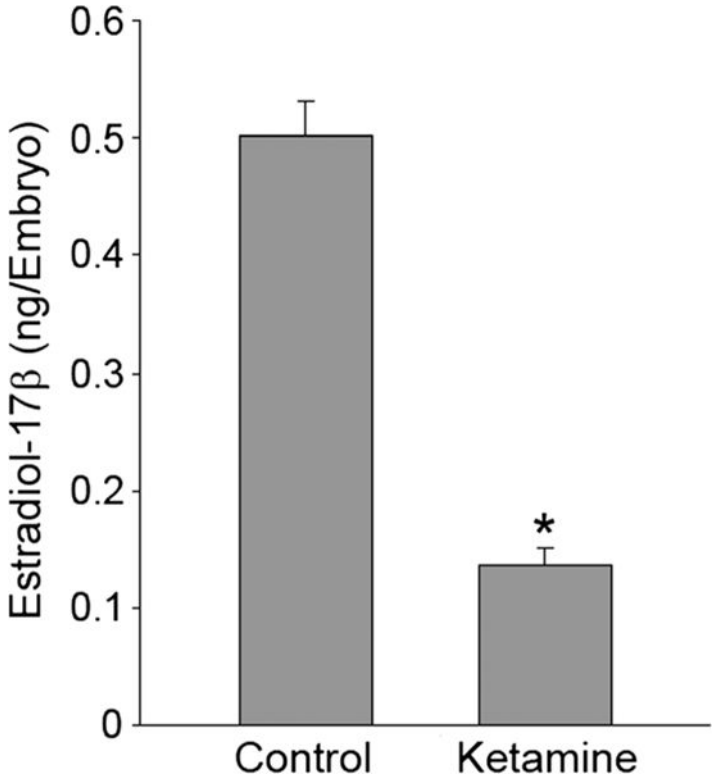

Figure 2.

Effect of ketamine on E2 levels in zebrafish embryos. Embryos at 48 h post fertilization (hpf) were exposed to 2 mM ketamine for 24 h (static exposure). Post exposure, embryos were washed extensively with quick changes of embryo water three times. Reverse-phase high-performance liquid chromatography (HPLC) was employed to measure estradiol-17β (E2) levels in the embryos ($n = 50$ per group). Data from three separate experiments were averaged and are presented as mean ± SD. Student's t test was used to determine significance $P < 0.05$ (*).

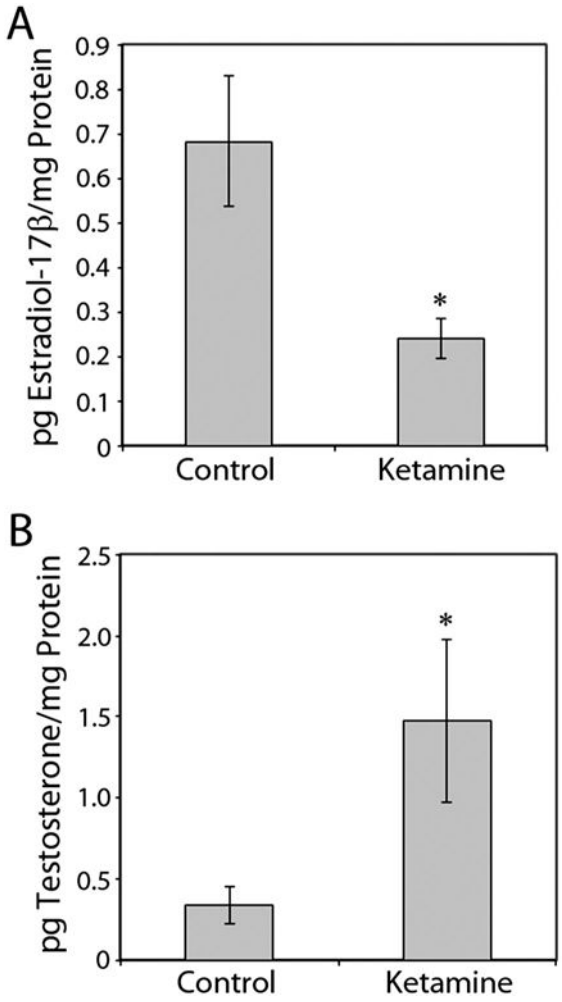

Figure 3.

Determination of the effects of ketamine on E2 (estradiol-17β) and testosterone levels in zebrafish embryos. Embryos (n = 125 per sample) at 48 h post fertilization (hpf) were treated either with or without ketamine (2 mM) for 24 h. Estimations of E2 (A) and testosterone (B) levels were performed by ELISA as described in the Materials and Methods. Data from three separate experiments were averaged and are presented as the mean ± SD. Student's t test was used to determine significance $P < 0.05$ (*).

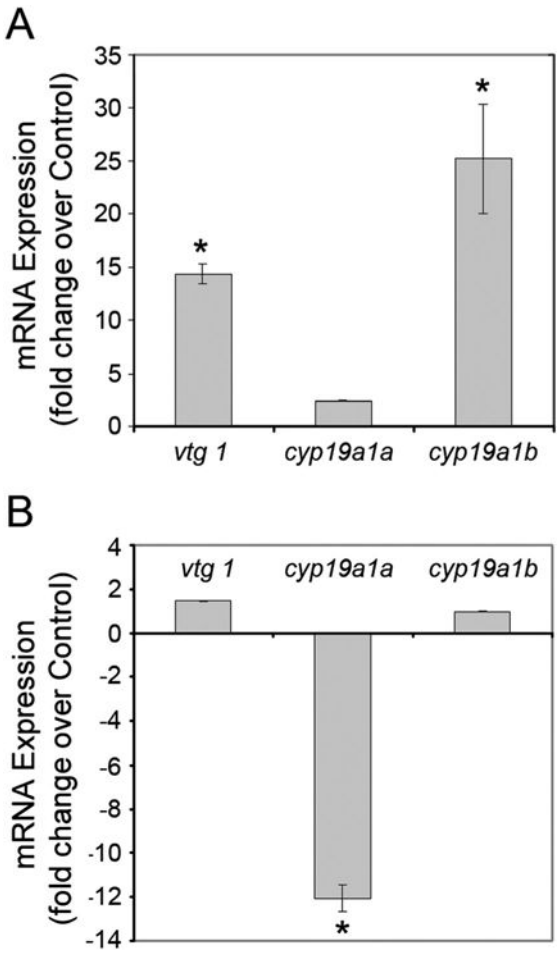

Figure 4.

Effects of E2 (A) and ketamine (B) on the expression of *vtg 1*, *cyp19a1a* and *cyp19a1b* genes in zebrafish embryos. Embryos at 48 h post fertilization (hpf) were treated with either 1 nM estradiol-17β (E2) or 2 mM ketamine for 24 h (static exposure). Total RNA was isolated from the embryos. After first strand cDNA synthesis from the RNA, quantitative real-time polymerase chain reaction (RT-qPCR) was performed. The 2-ΔΔCT method was used to determine relative gene expression. The GAPDH gene was the internal control for all RT-qPCR experiments. Data were averaged and shown as normalized (fold change over control) gene expression ± SD. One-way ANOVA and Holm Sidak pair-wise multiple comparison post-hocs (Sigma Stat 3.1 for analysis) were used to determine statistical significance with $P < 0.05$ (*).

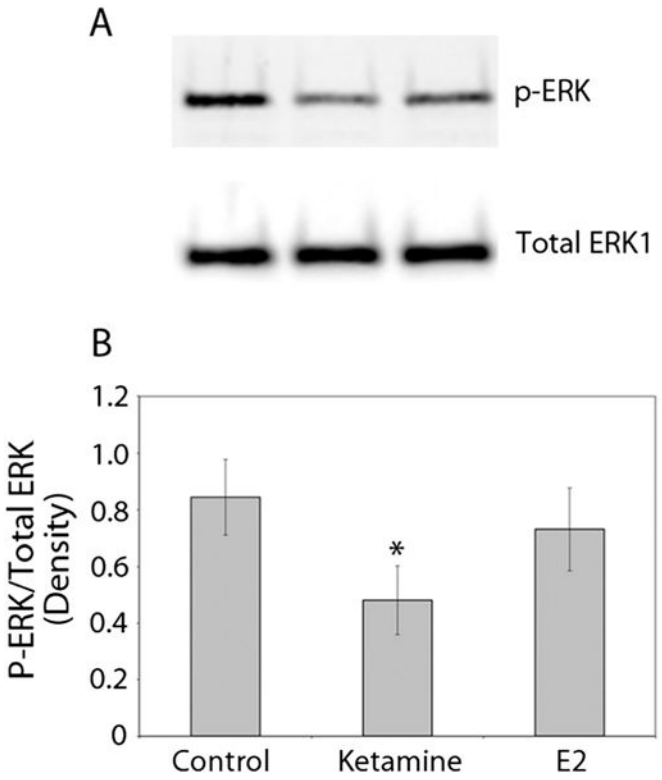

Figure 5.

Effect of ketamine and estradiol-17β (E2) on activated ERK (phosphorylated ERK or p-ERK) levels in zebrafish embryos. Ketamine (2 mM) or E2 (1 nM) treatment of 48 h post fertilization (hpf) embryos was for 24 h (static exposure). (A) Western blot analysis conducted on whole-embryo lysates (one embryo/lane) shows activated ERK (p-ERK) and total ERK levels (on the same blot used after stripping). (B) Densitometric analysis (presented as the ratio of the densities of p-ERK to total ERK) of the Western blots. Data from three separate experiments were averaged and shown as the mean ±SD. Student's *t* test was used to compare the densitometric values of the P-ERK/Total ERK of the control and the respective treated groups. *Significance was set at $P < 0.05$.

Table 1

List of primers used in quantitative real-time polymerase chain reaction (RT-qPCR) assays

Gene	Forward primer	Reverse primer
cyp19a1a	5′-TCTGCTTCAGAAGATTCATAAATACTTT-3′	5′-CCTGCAACTCCTGAGCATCTC-3′
cyp19a1b	5′-AAAGAGTTACTAATAAAGATCCACCGGTAT-3′	5′-TCCACAAGCTTTCCCATTTCA-3′
vtg 1	5′-CTGCGTGAAGTTGTCATGCT-3′	5′-GACCAGCATTGCCCATAACT-3′
GAPDH	5′-GATACACGGAGCACCAGGTT-3′	5′- GCCATCAGGTCACATACACG-3′