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L-Carnitine rescues ketamine-induced attenuated heart rate and MAPK (ERK) activity in zebrafish embryos

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Abstract

Ketamine, an antagonist of the N-methyl-D-aspartate (NMDA)-type glutamate receptors, is a pediatric anesthetic. Ketamine has been shown to be neurotoxic and cardiotoxic in mammals. Here, we show that after 2 h of exposure, 5 mM ketamine significantly reduced heart rate in 26 h old zebrafish embryos. In 52 h old embryos, 1 mM ketamine was effective after 2 h and 0.5 mM ketamine at 20 h of exposure. Ketamine also induced significant reductions in activated MAPK (ERK) levels. Treatment of the embryos with the ERK inhibitor, PD 98059, also significantly reduced heart rate whereas the p38/SAPK inhibitor, SB203580, was ineffective. Ketamine is known to inhibit lipolysis and a decrease of ATP content in the heart. Co-treatment with L-carnitine that enhances fatty acid metabolism effectively rescued ketamine-induced attenuated heart rate and ERK activity. These findings demonstrate that L-carnitine counteracts ketamine's negative effects on heart rate and ERK activity in zebrafish embryos.

Keywords

Heart rate; Ketamine; Zebrafish; L-Carnitine; MAPK/ERK; SAPK

1. Introduction

Ketamine is a dissociative anesthetic introduced in the 1960s, which produces anesthesia, analgesia, suppression of fear and anxiety, and amnesia. Ketamine is an agent commonly used in pediatric anesthesia, acting primarily through blockade of N-methyl-D-aspartate (NMDA)-type glutamate receptors to provide sedation/analgesia to children for painful procedures [1]. Studies in humans and animals have shown that ketamine has a direct negative [2–5] or an indirect positive [6–8] inotropic effect on myocardial contractility. It is well known that clinically ketamine enhances myocardial contractility by central nervous system-mediated sympathomimetic stimulation, baroreceptor activation, and inhibition of

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Conflict of interest

The authors declare that there are no conflicts of interest.

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neuronal uptake of catecholamines [9]. In contrast, in critically ill patients ketamine has been shown to be a cardiac depressant and its actions in these subjects are related to their dysfunctional sympathomimetic systems [10]. Ketamine also acts as a cardiac depressant in dogs after pharmacological blockade of the autonomic nervous system [11]. In opiate-dependent patients, ketamine was not associated with significant changes in heart rate [12] and a similar lack of effect was observed in patients injected with 10 µg/kg [13]. In isolated human muscle strips, a low dose of ketamine (73 µM) increased contractility and relaxation while at a higher dose (730 µM) a direct inotropic action was observed [14]. Ketamine increases heart rate by 34% in patients with ischemic heart disease [15]. Despite a large number of publications on hemodynamic responses to ketamine, its mode of action on cardiac function is still not clear.

Although most anesthetics are known to decrease heart rate, ketamine has been shown to increase heart rate and systemic and pulmonary vascular resistance that results in increased systemic and pulmonary blood pressure [16]. However, a different effect of ketamine on heart rate has been documented in several mammalian species. Ketamine reduces heart rates in rats [17] and guinea pigs [18]. In rhesus monkeys, extended intravenous infusions of ketamine result in decreased heart rates in pregnant females and in PND (post natal day) 5 and PND 35 infants [19] but in older humans (50–60 years of age); intravenous ketamine (1 mg/kg) does not affect heart rate [20].

There are ten NMDA receptor (NR) subunits in zebrafish with five subtypes, each containing two paralogous genes [21]. Only one paralog of the NR2B is expressed in the heart at 48 hpf. Based on reported observations that ketamine affects cardiac function in mammals including humans, primates and rodents, the goal of this study was to test whether similar effects manifest in zebrafish, an emerging alternate vertebrate animal model for drug screening and drug safety assessments.

In adult zebrafish, 0.8% (29 mM) ketamine is an effective anesthetic dose and 0.2% (7.25 mM) is a subthreshold dose. At the subthreshold (subanesthetic) dose, zebrafish show a variety of abnormal behaviors, such as altered gill movement, stress responses and circling behavior [22]. Zebrafish larvae (6-day old) treated with 0.1–3.0 mM ketamine for 20 min show altered sensorimotor gating [23]. In our study using zebrafish embryos, we used ketamine over the dose range of 0.5 (0.14%) to 10 mM (0.27%) to determine its dose-dependent effect on heart rate.

Several pharmacological agents that are able either directly or indirectly to inhibit mitochondrial fatty acid oxidation (etomoxir, oxfenicine, dichloroacetate, trimetazidine, ranolazine, pyruvate, malonyl-CoA decarboxylase inhibitors, L-carnitine), have been shown to improve cardiac function in experimental and clinical studies of cardiac ischemia and failure [24,25]. L-Carnitine is an L-lysine derivative and its main role lies in the transport of long chain fatty acids into mitochondria to enter the β-oxidation cycle [26]. Another important property of this agent is the neutralization of toxic acylCoA production in the mitochondria [27]. Stimulation of glucose consumption by L-carnitine has also been shown in cardiomyocytes [28]. L-Carnitine preserves the mechanical function of ischemic swine hearts perfused in the presence of free fatty acids [29]. A significant improvement in

mechanical recovery, measured as the heart rate-peak systolic pressure product, was observed in L-carnitine-perfused post-ischemic rat hearts along with a two-fold increase in glucose oxidation rate compared to controls [30] and in diabetic rat hearts [31,32]. There is a substantial literature describing the clinical efficacy of L-carnitine in patients affected by heart disease [33–36]. Moreover, L-carnitine has been shown to ameliorate the ketamine-induced behavioral alterations and body weight deficits in developing rats [37], in addition to having neuroprotective abilities [38].

Through the MAPK (mitogen-activated protein kinase) signaling system, cells transduce extracellular stimuli into intracellular responses [39]. There are three major sub-groups that include extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and c-jun-N-terminal kinase (JNK). P38 MAPK is a subfamily of the stress-activated MAPKs (SAPKs) [40–42]. In isolated rat hearts, drugs like ketamine and imipramine have been shown to activate the MAPK/ERK (extracellular signal-regulated kinase), which is accompanied by an increase in Mg^{2+} that results in decreased heart rate [18,43]. Here, we explored the role of SAPK (stress-activated protein kinase)/p38 kinase since ketamine activation of the p38 kinase in isolated guinea pig hearts has also been linked to cardiac depression [18]. Additionally, we examined whether ketamine affected heart rate and ERK activation in zebrafish embryos and whether L-carnitine interfered with ketamine's effects.

2. Materials and methods

2.1. 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 8:30 AM. For in-system breeding, crosses of males and females were set up the previous day with partitions that were taken off the following morning at the time of light onset at 8:30 AM. Fertilized zebrafish embryos were collected from the bottom of the tank as soon as they were laid. The eggs/embryos were placed in Petri dishes and washed thoroughly with buffered egg water (reverse osmosis water containing 60 mg sea salt (Crystal Sea®, Aquatic Eco-systems, Inc., Apopka, FL, USA) per liter of water (pH 7.5) and then allowed to develop in an incubator at 28 °C for further experiments.

2.2. Reagents

Ketamine, L-carnitine and the monoclonal anti-activated MAPK (diphosphorylated ERK1/2) antibody were purchased from Sigma (St. Louis, MO, USA). The p38 kinase inhibitor, SB203580 and the ERK (MAPK) inhibitor, PD 98059 were purchased from Promega Corp. (Madison, WI, 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 L-carnitine

Manual dechoriation of the embryos began at 24 h of development. For treatment with ketamine, three replicates of 26 h and 52 h dechorionated embryos were used. Each replicate consisted of 10 embryos per experimental group (total $n = 30$) that were placed in individual wells of six-well plates. Ketamine and L-carnitine treatments at various doses continued for 2 h or 20 h. Untreated control groups of 10 embryos per replicate were examined in parallel.

2.4. Scoring heart rate

Live images of the embryos in the six-well plates were acquired using an Olympus SZX 16 binocular microscope and DP72 camera and heart rate of each embryo was measured by visually counting (blinded) ventricular beats per minute on the live video using a timer (stop watch). Data for each replicate ($n = 10$) were used to calculate the mean and SD independently. The statistical significance of the effects of the various treatments on heart rate was determined by one-way ANOVA (SigmaStat) using Holm–Sidak pair-wise multiple comparison post-hoc analysis.

2.5. Electrophoresis and Western blot analysis

Following treatment with ketamine or L-carnitine or L-carnitine plus ketamine, untreated (Control) and treated embryos were prepared for immunoblotting (Western blot) through hand-homogenization (2 embryos 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 were run in 4–20% gradient SDS-polyacrylamide gels purchased from Bio-Rad (Hercules, CA) 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 anti-mouse IgG diluted in blocking buffer (1:2500; Sigma–Aldrich, St. Louis, MO). After development for chemiluminescent signals, the same membrane was stripped from the bound antibodies using the stripping buffer (Thermo Scientific, Rockford, IL) and reprobed with the total ERK1 anti-rabbit IgG (1:500; Santa Cruz Biotech., CA). Advanced ECL Western Blotting Substrate (GE Healthcare, Piscataway, NJ) was used for detecting HRP-conjugate bound secondary antibodies. The volume equivalent of two embryos was used at each time point analyzed. For quantification, densitometry of the immunoblot signals was performed using ImageJ (NIH, USA).

3. Results

3.1. Ketamine induces changes in heart rate in zebrafish embryos

In order to assess the effects of ketamine on heart rate, we used zebrafish embryos (Fig. 1). In 26 hpf (hours post-fertilization) embryos, a 2-h treatment with ketamine did not alter heart rate at concentrations up to 2 mM (0.055%). However at concentrations of 5 and 10 mM, ketamine caused a significant decrease ($P < 0.001$) in heart rate, although there was no

significant difference between the effects of these two doses (Fig. 1A). In 52 hpf embryos however, a concentration of 1 mM ketamine was sufficient to lower the heart rate significantly after 2 h of treatment (Fig. 1B). At 2 mM ketamine further decreased heart rate and at 5 mM there was a drastic reduction in heart rate with no further decrease occurring at the higher 10 mM concentration (Fig. 1B). These results, along with the results from the 26 hpf embryos indicated that 5 mM dose was effecting the maximal heart rate response. Further support for this observation came from findings from the 20 h exposure of the 52 hpf embryos in which there was a clear dose-dependent decrease in heart rate (significant effects at doses of 0.5 mM and higher) with no further change noted when the dose was increased from the 5 to the 10 mM concentrations (Fig. 1C).

3.2. Effect of the SAPK/p38 kinase inhibitor, SB203580 on zebrafish embryo heart rate

In order to determine the relevant signaling pathways that participate in ketamine-induced changes in heart rate, we focused on the p38 kinase that has been shown to be activated in perfused guinea pig hearts upon ketamine treatment [18]. A member of the SAPK family, p38 kinase activity is inhibited by a synthetic inhibitor, SB203580. In zebrafish, others have used up to 100 μ M SB203580 in zebrafish embryos to successfully block p38 kinase activity [44,45]. In our study, a 2 h treatment (static exposure) with 50 μ M SB203580 did not affect heart rate in 52 hpf embryos (Fig. 2A). Increasing the SB203580 concentration to 100 μ M and extending the treatment for 4 h (static exposure) also did not produce any effects (data not shown). At 5 mM ketamine reduced heart rate compared to controls (DMSO-treated) and SB203580 did not alter the effect of ketamine (Fig. 2A). These results suggest that the ketamine-induced change in heart rate may not involve the SAPK/p38 kinase pathway.

3.3. Effect of the MAPK/ERK inhibitor PD 98059 on zebrafish embryo heart rate

In guinea pig hearts, ketamine has been shown to activate ERK (MAPK) [18]. In our study, we first sought to determine whether the synthetic ERK inhibitor, PD 98059 affects heart rate in zebrafish. Embryos at 52 hpf were treated with either 5 mM ketamine or 5 mM ketamine along with 50 μ M PD 98059 [this concentration was chosen based on a previous report by Tallafus and Eisen [45] or 50 μ M PD 98059 alone. After 2 h of treatment (static exposure), not only did PD 98059 significantly reduce the heart rate in the embryos compared to the control (DMSO-treated), but also significantly added to the reduction in heart rate caused by ketamine (Fig. 2B). Increasing the treatment for 4 h did not produce any difference in effects (data not shown). These results suggest that either PD 98059 negatively affected heart rate by a mechanism completely independent of that of ketamine or it might exert its effect via the same pathways as ketamine. Either way, this part of the study addressed the fundamental question about whether ERK is involved in regulating the heart rate in zebrafish and demonstrated that ERK inhibition decreases heart rate.

3.4. Effect of ketamine on the ERK/MAPK activation

Next, we sought to determine whether ketamine also induced a decrease in ERK activity. First, 52 h embryos were treated for 20 h (static exposure) with 2 mM ketamine (0.5, 2.0 and 5.0 mM). In order to determine the level of activated ERK/MAPK associated with this effect, immunoblots using phospho-MAPK (P-ERK) antibody of whole embryo lysates were obtained. The results revealed a significant reduction in P-ERK in ketamine-treated embryos

indicating that ketamine negatively affected ERK/MAPK activity (Fig. 3A, upper panel). Immunoblots, using a total ERK1 antibody, were used to determine equal loading (Fig. 3A, lower panel). Quantification of the activated ERK (P-ERK) level was performed by densitometric analysis to obtain the ratio of P-ERK/total ERK1 for comparison (Fig. 3B). Next we explored whether the down-regulation ERK/MAPK activity upon ketamine treatment was dose-dependent. Thus 52 h embryos were treated for 20 h (static exposure) with ketamine at doses 0.5, 2.0 and 5.0 mM. Immunoblots showed that the down-regulation of ERK/MAPK activity upon ketamine exposure occurred in a dose-dependent manner (Fig. 3C and D). These results showed that at 20 h when ketamine reduced heart rate, MAPK activity was also reduced in a dose-dependent manner suggesting a link between MAPK activity and heart rate.

3.5. Effect of L-carnitine on ketamine-induced attenuated heart rate in zebrafish embryos

Among the compounds that appear to have a neutralizing effect on ketamine-induced toxicities, such as neuronal apoptosis in the developing rat frontal cortex, is L-carnitine [38], a naturally occurring amino acid that induces fatty-acid oxidation. The mechanism underlying the effect of L-carnitine to inhibit such neurotoxicity is not clear. On the other hand, ketamine is known to induce blockade of lipid oxidation in rat heart [46]. We explored whether L-carnitine could counteract this effect and rescue the heart rate. While 0.1 mM L-carnitine did not affect the ketamine-induced decrease in heart rate, doses of 0.5 and 1.0 mM L-carnitine were equally effective in completely inhibiting that effect (Fig. 4A). From these data we chose to use 1 mM L-carnitine for subsequent experiments. While ketamine decreased heart rate after a 20-h treatment to almost one third of the control rate, co-treatment with either 0.5 or 1 mM L-carnitine completely blocked this effect. Treatment with 1 mM L-carnitine alone had no effect on heart rate (Fig. 4B).

3.6. Effects of L-carnitine on ketamine-induced attenuation of ERK/MAPK activation

Based on these observations, we sought to determine whether restoring MAPK activity to normal levels would also block the ketamine-induced reduction in heart rate.

Since our previous experiments demonstrated that the ketamine-induced decrease in heart rate was associated with a concomitant reduction in ERK/MAPK activity, we sought to determine how these two outcomes might be linked and if the inhibition of ketamine's effects by L-carnitine would occur with a simultaneous reversion of activated ERK/MAPK (P-ERK) levels back to normal. Accordingly, 52 h embryos were treated for 20 h with 2 mM ketamine or 1 mM L-carnitine or 2 mM ketamine plus 1 mM L-carnitine and the heart rates were scored (Fig. 5A). In these groups, as expected, ketamine-induced decrease in heart rate was inhibited in presence of 1 mM L-carnitine. Post-treatment embryo lysates were processed for immunoblotting. Immunoblots probed with the phospho-MAPK (P-ERK) antibody revealed a significant reduction (~50%) in P-ERK in ketamine treated embryos compared to the untreated controls, while there was no significant change in the L-carnitine- or ketamine plus L-carnitine-treated groups (Fig. 5B, upper panel). Immunoblotting with the total ERK1 antibody was used to determine equal loading (Fig. 5B, lower panel). Quantification of the activated ERK (P-ERK) level was performed using densitometric analysis to obtain the ratio of P-ERK/total ERK1 for comparison (Fig. 5C). These results

indicated that L-carnitine's inhibition of ketamine's effect to decrease heart rate is linked to the regulation of MAPK (ERK) activity. Since L-carnitine treatment alone does not accelerate heart rate nor enhance ERK/MAPK activity, it is presumed that L-carnitine somehow interferes with the pathway(s) through which ketamine acts to reduce ERK/MAPK activity.

4. Discussion

The effect of ketamine, an NMDA receptor antagonist and a pediatric anesthetic, on heart rate has been reported for a variety of mammals including humans. Ketamine reduces heart rates in rats [17], guinea pigs [18], and pregnant and infant monkeys (5 and 35 days old) [19]. However, in older humans (50–60 years of age), intravenous ketamine does not affect heart rate [20]. In intact isolated rabbit heart, ketamine has dual actions: at low concentrations it mildly increases cardiac contractility whereas at high concentrations it profoundly depresses the myocardium [5]. Our results show that in 26 h embryos, 2 h exposures to ketamine at concentration up to 2 mM were ineffective in decreasing heart rate whereas 5 and 10 mM doses were very effective with no differences between the latter two doses. Based on previous studies of ketamine using 6 dpf zebrafish larvae (concentrations from 0.1 to 3 mM) [23], these doses are relatively high and, thus, may be exerting non-specific effects. If that were true, one interpretation of our findings could be that the 26 h zebrafish embryo heart does not respond to ketamine in any specific fashion. At 24 h no NMDA receptors (NR) are expressed in the zebrafish heart [21]. In 52 h embryos a 2 h exposure to 1 mM ketamine was effective in significantly lowering the heart rate indicating that the heart could be much more sensitive at this stage of development than in 26 h embryos. This difference in sensitivity could be related to the differential expression of NR expression when NR2B subunit is expressed exclusively in the heart at 48 hpf [21]. This differential expression of the NR subtypes at different developmental stages in the zebrafish may, therefore, represent an ideal system for determining the specific pathway(s) underlying ketamine's action in vivo.

Since ketamine-induced cardiac depression in isolated guinea pig hearts has been shown to be accompanied by activation of SAPK/p38 kinase and MAPK/ERK1/2 [18], and cardiac-specific activation of p38 kinase in vivo markedly attenuates cardiac contractility in mice [47], we next examined these kinases in ketamine-treated zebrafish. Our results were in contrast to those findings in the guinea pig heart preparations. While p38 kinase inhibitor did not affect heart rate in zebrafish embryos, ketamine reduced ERK activity instead of activating it. These differences could be related to several factors. The zebrafish embryo is an intact organism in which the heart beat is observed in its native environment (water) rather than in isolation in a culture medium [18]. Being embryonic may impart different characteristics than are typical of adult heart. And, it is known that there are species differences in cardiovascular responses to ketamine [48]. For example in Leghorn hens ketamine decreased heart rate at a relatively low dose [49]; in rabbits ketamine exposure caused a “slow normal” heart rate [50] and in rats, it stimulated cardiovascular function [51]. In new born infants, ketamine at a dose of 2 mg/kg significantly reduces heart rate compared to the placebo [52]. The zebrafish embryo response to ketamine with regard to heart rate is concordant with that of the human infants [52] and non-human primate PND5 and 35 infants

[19]. Differential effects of ketamine on heart rate in new born and adult humans has been attributed to the poorly developed sarcoplasmic reticulum of the neonatal myocardium [53], which appears to be more dependent on the entry of extracellular calcium rather than the release of calcium from intracellular sarcoplasmic reticulum [54]. It has also been shown that the p38 kinase inhibitor, SB 203580, reverses stress-induced myocardial dysfunction in vivo in rats [55]. In zebrafish, we presume that the ketamine-induced decrease in heart rate is not stress-related and therefore, may be independent of the stress-inducible p38 kinase pathway.

In zebrafish embryos, ketamine induced a down-regulation of ERK activity as demonstrated by a lower level of activated (P-ERK) levels in immunoblots. The ERK inhibitor, PD 98059 alone, unlike the p38 kinase inhibitor, SB 203580 also significantly decreased heart rate. These results contrast with those from ketamine-treated guinea pig cardiomyocytes, in which ketamine induced ERK/MAPK activation [18]. However, it has also been reported that ketamine suppresses ERK activation in the brains of 5 day old mice [56]. ERK is expressed in almost all cell types and whether the effect of its inhibitor, PD 98059, is direct or indirect on zebrafish embryo heart rate remains to be examined.

The role of NMDA receptors in regulating the MAPK pathway has been extensively investigated over the past several years. A large number of studies have documented a profound increase in activated ERK in response to pharmacological activation of NMDA receptors on neurons [56–58]. As for mechanisms transmitting NMDA receptor signals to MAPKs, there is a Ca^{2+} -sensitive pathway associated with this activity [56–59]. Briefly, activation of the Ca^{2+} permeable NMDA receptors results in an increase in Ca^{2+} influx. The Ca^{2+} signals then induce several Ca^{2+} -dependent kinases including ERK via calmodulin-binding exchange factor GRF2 and Ras [60,61]. Ketamine decreases Ca^{2+} release from intracellular stores of rat ventricular myocytes [62]. There are also reports that the cardiac depressive effects of ketamine could be caused by interference with Ca^{2+} influx [7,14]. In the present study, L-carnitine neutralized the ketamine-induced decrease in heart rate and returned the ketamine-induced decrease in activated ERK (P-ERK) levels back to normal, suggesting that L-carnitine may have altered cellular Ca^{2+} transport in doing so, since palmitoyl carnitine is known to be an endogenous promoter of calcium efflux from rat heart mitochondria [63] and acylcarnitines increase calcium concentrations in the cytoplasm of rat ventricular cardiomyocytes [64]. In patients with mild diastolic heart failure L-carnitine improves their symptoms [65]. Differential effects of ketamine and L-carnitine are that ketamine induces a blockade of lipid oxidation and a decrease in ATP content in rat heart [45] whereas L-carnitine facilitates fatty-acid oxidation to support ATP production [62]. Whether such counteracting effects regulate the heart rate in zebrafish embryos remain to be determined. L-Carnitine also reverses the reduced heart rate induced by verapamil, an L-type Ca^{2+} channel blocker, in the zebrafish embryos (our unpublished data) indicating that L-carnitine may be regulating heart rate by modulating intracellular Ca^{2+} levels. Based on these information, a potential mechanism for ketamine's and L-carnitine's effects on MAPK/ERK in zebrafish is summarized in Fig. 6.

In conclusion, we show that ketamine decreases heart rate in zebrafish in a dose-dependent manner and L-carnitine reverses this effect although L-carnitine itself has no effect on the

heart rate. Ketamine induces decrease in activated ERK (P-ERK) levels and L-carnitine inhibits this suppression. Further experiments will be required to elucidate the exact mechanism(s) of how ketamine and L-carnitine modulate ERK activity and whether ERK is directly linked to the regulation of heart rate in zebrafish.

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Fig. 1. Effect of ketamine on heart rate in zebrafish embryos. Embryos were exposed to various doses (0.5–10 mM) of ketamine. Each exposure (static exposure) group consisted of 10 embryos and the experiment was repeated three times. Heart rate was measured in beats per minute and the data from all the embryos in a group were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. *Significance was set at $P < 0.05$. Heart rates are shown for (A) 2 h exposure of the 26 hpf (hours post fertilization) embryos, (B) 52 hpf embryos (B), and (C) 20 h exposure for the 52 hpf embryos.

Fig. 2.

Effects of MAPK and SAPK signaling inhibitors on heart rate in zebrafish embryos. (A) The SAPK/p38 kinase inhibitor, SB 203580 (50 μ M), has no effect on the heart rate of 52 hpf zebrafish embryos. Treatment was for 2 h ($n = 10$, for each group/experiment). Ketamine dose was 5 mM (static exposure). Control groups were treated with equal volumes of DMSO (0.1%). The experiment was repeated three times. Heart rate was measured in beats per minute and data are presented as the mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. *Significance was set at $P < 0.05$. (B) The MAPK/ERK inhibitor, PD98059 (50 μ M), reduces heart rate in 52 hpf zebrafish embryos. Treatment was for 2 h ($n = 10$, for each exposure group/experiment). Ketamine dose was 5 mM (static exposure). Control groups were treated with equal volumes of DMSO (0.25%). The experiments was repeated three times. Heart rate was measured in beats per minute and data are shown as mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. */**Significance was set at $P < 0.05$.

Fig. 3. Effect of ketamine on activated ERK (phospho-ERK) levels in zebrafish embryos. Ketamine treatment of 52 hpf larvae was for 20 h (static exposure). (A) Western blot analysis conducted on whole-embryos (2 mM ketamine-treated) lysates shows activated phospho-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 phospho-ERK to total ERK1) confirms a significant decrease in ketamine-induced phospho-ERK levels. Data from replicates (three separate experiments) were averaged and shown as mean $\pm$ SD. Student's *T* test was used to compare the densitometric values of the P-ERK/Total ERK1 of the control and the treated groups. *Significance was set at $P < 0.05$. (C) Western blot analysis conducted on whole-embryos (treated with 0.5, 1.0, and 2.0 mM ketamine) lysates shows activated phospho-ERK (P-ERK) and total ERK levels (on the same blot used after stripping). (D) Data from densitometric analysis are presented as the ratio of the relative densities of phospho-ERK to total ERK1. Data from replicates (three separate experiments) were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the densitometric values (relative densities) of the P-ERK/Total ERK1 of the control and the treated groups. *Significance was set at $P < 0.05$.

Fig. 4.

Dose-dependent effects of L-carnitine on ketamine-induced decrease in heart rate. L-Carnitine inhibits the ketamine-induced decrease in heart rate in zebrafish embryos. Alongside the control, untreated group embryos at 52 hpf ($n = 10/\text{group}$) were treated with 2 mM ketamine in presence or absence of L-carnitine (0.1, 0.5, and 1.0 mM). One group received 1 mM L-carnitine only. All the exposures were static and were for either (A) 2 h or (B) 20 h. The experiment was repeated three times. Data are presented as the mean $\pm$ SD. One-way ANOVA was used to compare the heart rates between the control and the treated groups. *Significance was set at $P < 0.05$.

Fig. 5. Correlation of the effects of ketamine and L-carnitine on heart rate and ERK activity in zebrafish embryos. (A) L-Carnitine rescues ketamine induced down regulation of heart rate. Embryos at 52 hpf ($n = 10/\text{group}$) were treated with 2 mM ketamine or 1 mM L carnitine or 2 mM ketamine plus 1 mM L-carnitine alongside the control untreated group. Exposure was for 20 h (static exposure). (B) L-Carnitine rescues ketamine induced down-regulation of activated ERK. Embryos at 52 hpf were treated with 2 mM ketamine or 1 mM L carnitine or 2 mM ketamine plus 1 mM L-carnitine alongside the control untreated group. Exposure was for 20 h (static exposure). Western blot analyses were conducted on whole-embryo lysates (2 embryos equivalent/lane) and show activated phospho-ERK (P-ERK) and total ERK1 levels (on the same blot used after stripping). (C) Densitometric analysis (presented as the ratio of

the densities of P ERK to total ERK1) confirms a significant decrease in ketamine induced phospho-ERK levels whereas L-carnitine alone or ketamine plus L-carnitine treatment did not affect activated P-ERK levels. Data from three separate experiments were averaged and shown as mean $\pm$ SD. One-way ANOVA was used to compare the densitometric values of the P-ERK/Total ERK1 of the control and the treated groups. *Significance was set at $P < 0.05$.

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Fig. 6.

Schematic presentation of a potential mechanism of ERK/MAPK modulation by ketamine and L-carnitine. NMDA receptors are Ca^{2+} permeable. In presence of ketamine, this property of the NMDA receptor is lost. NMDA receptor permeable Ca^{2+} is known to couple CaM (calmodulin) to GRF2 which then activates Ras, an upstream activator of ERK/MAPK. In the absence of extracellular Ca^{2+} entry into the cells via the NMDA receptors challenged with ketamine, addition of L-carnitine could generate mitochondrial release of Ca^{2+} . This intracellular Ca^{2+} could couple CaM (calmodulin) and GRF2 to activate Ras followed by ERK/MAPK activation.