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Ketamine induces motor neuron toxicity and alters neurogenic and proneural gene expression in zebrafish

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Abstract

Ketamine, a noncompetitive antagonist of *N*-methyl-D-aspartate-type glutamate receptors, is a pediatric anesthetic that has been shown to be neurotoxic in rodents and nonhuman primates when administered during the brain growth spurt. Recently, the zebrafish has become an attractive model for toxicity assays, in part because the predictive capability of the zebrafish model, with respect to chemical effects, compares well with that from mammalian models. In the transgenic (*hb9:GFP*) embryos used in this study, green fluorescent protein (GFP) is expressed in the motor neurons, facilitating the visualization and analysis of motor neuron development *in vivo*. In order to determine whether ketamine induces motor neuron toxicity in zebrafish, embryos of these transgenic fish were treated with different concentrations of ketamine (0.5 and 2.0 mM). For ketamine exposures lasting up to 20 h, larvae showed no gross morphological abnormalities. Analysis of GFP-expressing motor neurons in the live embryos, however, revealed that 2.0 mM ketamine adversely affected motor neuron axon length and decreased cranial and motor neuron populations. Quantitative reverse transcriptase-polymerase chain reaction analysis demonstrated that ketamine down-regulated the motor neuron-inducing zinc finger transcription factor *Gli2b* and the proneural gene *NeuroD* even at 0.5 mM concentration, while up-regulating the expression of the proneural gene *Neurogenin1* (*Ngn1*). Expression of the neurogenic gene, *Notch1a*, was suppressed, indicating that neuronal precursor generation from uncommitted cells was favored. These results suggest that ketamine is neurotoxic to motor neurons in zebrafish and possibly affects the differentiating/differentiatedneurons rather than neuronal progenitors. Published 2011. This article is a US Government work and is in the public domain in the USA.

Keywords

neurotoxicity; ketamine; motor neuron; transgenic zebrafish; gene expression

INTRODUCTION

Ketamine, a pediatric anesthestic, 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). Rodent research has shown that ketamine

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can induce apoptosis when administered in high doses and/or for prolonged periods during susceptible periods of development (Lahti *et al.*, 2001; Larsen *et al.*, 1998; Malhotra *et al.*, 1997; Maxwell *et al.*, 2006). Although it is clear that ketamine causes neuronal cell death in rodent models when given repeatedly during the brain growth spurt period (Ikonomidou *et al.*, 1999; Wang *et al.*, 2006), it has only recently been demonstrated that a similar phenomenon also occurs in primates (Haberny *et al.*, 2002; Slikker *et al.*, 2007; Wang *et al.*, 2006). However, plasma ketamine levels associated with a light surgical plane of anesthesia in neonatal monkeys (postnatal days 5–6) were 3–5 times higher (Slikker *et al.*, 2007) than those typically observed in humans (Clements and Nimmo, 1981; Grant *et al.*, 1981).

Earlier rodent and monkey studies suggest that limiting doses and durations of exposure reduces the potential for neurodegeneration caused by ketamine and other NMDA receptor antagonists (Ikonomidou *et al.*, 1999, 2001; Jevtovic-Todorovic *et al.*, 2000; Olney *et al.*, 2002, 2004; Pohl *et al.*, 1999; Slikker *et al.*, 2007). Although the exact mechanisms underlying the neurotoxicity induced by ketamine are not known, the window of vulnerability to the neuronal effects of anesthetics is restricted to the period of rapid synaptogenesis, also known as the brain growth spurt (Slikker *et al.*, 2007).

In addition to 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). 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). The zebrafish has also been used as a model for other areas of research such as aging (Gerhard, 2007; Gerhard and Cheng, 2002), neurological diseases (Bretaud *et al.*, 2004), drug addiction (Ninkovic and Bally-Cuif, 2006), and other behavioral studies (Fetcho and Liu, 1998; Miklosi and Andrew, 2006; Salas *et al.*, 2006).

NMDA receptor channels are heteromeric complexes consisting of essential NR1 subunits and one or more regulatory NR2 subunits (NR2A/B/C/D; Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992). Regulation of NMDA receptor channel activity is critical for normal neural development (Cull-Candy *et al.*, 2001), learning and memory (Abel and Lattal, 2001), and the pathological processes associated with stroke (Meldrum, 1990). There are 10 NMDA receptor subunits found in zebrafish (Cox *et al.*, 2005). These subunits fall into five subtypes, each containing two paralogous genes. There are two NMDAR1 genes (NR1.1 and NR1.2), and eight NMDAR2 genes, designated NR2A.1 and NR2A.2, NR2B.1 and NR2B.2, NR2C.1 and NR2C.2, and NR2D.1 and NR2D.2. The predicted sequences of the NR1 paralogs display 90% identity with the human protein. The NR2 subunits show less identity, differing most at the N- and C-termini. Both the NR1 genes are expressed embryonically, although in a nonidentical manner. NR1.1 is found in brain, retina and spinal cord at 24 h postfertilization (hpf). NR1.2 is expressed in the brain at 48 hpf but not in the spinal cord. NR2 developmental gene expression varies: both paralogs of the NR2A are expressed at 48 hpf in the retina; only one paralog of the NR2B is expressed at low levels in the heart at 48 hpf; no NR2C paralogs are expressed embryonically; and NR2D.1 is expressed in the forebrain, retina, and spinal cord at 24 hpf, whereas NR2D.2 is only found in the retina (Cox

et al., 2005). Studies based on immunohistochemistry with available antibodies have shown that NR2A subunits are expressed in primary motor neurons and axons in zebrafish larvae (Todd *et al.*, 2004). Expression of other subunits in both primary and secondary motor neurons remains to be determined.

In adult zebrafish, 0.8% ketamine is an effective anesthetic dose and 0.2% 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, qualitatively analogous to those observed in humans and rodents treated with various drugs (Zakhary *et al.*, 2011). Zebrafish larvae treated with 0.1–3.0 mM ketamine for 20 min show altered sensorimotor gating (Burgess and Granato, 2007). In our study on 28 hpf embryos, we used ketamine at 0.5 (0. 014%) and 2.0 mM (0.055%) concentrations for 20 h in order to study specifically its effects on motor neuron development.

In order to quantitate the effect of ketamine on motor neurons *in vivo*, *hb9*:green fluorescent protein (*hb9:GFP*) transgenic zebra-fish embryos were used, in which the promoter of the transcription factor hb9 that is found in developing motor neurons of both mammals (William *et al.*, 2003) and zebrafish (Cheesman *et al.*, 2004; Park *et al.*, 2004), drives GFP expression in motor neurons (Flanagan-Steet *et al.*, 2005).

For this study, several genes involved in neuronal development were selected for analysis. Gli2, the zinc finger transcription factors, play a role in several cellular lineages – the ventral neural precursors, cranial motor neurons, interneurons and dorsal sensory neurons (Ke *et al.*, 2005, 2008). Notch1a is a member of the Notch trans-membrane proteins that play a central role in the signaling events critical for nervous system development by lateral inhibition (reviewed in Lewis, 1996). In zebrafish, Notch-mediated lateral inhibition maintains a pool of neuronal precursors for later differentiation (Appel *et al.*, 1999) and transient inhibition of Notch signaling using the gamma-secretase inhibitor DAPT {*N*-[*N*-(3,5-difluorophenacetyl)-1-alanyl]-*S*-phenylglycine *t*-butyl ester} during early neurogenesis produces excess primary motor neurons and KA′ (Kolmer–Agduhr′) interneurons in zebrafish (Shin *et al.*, 2007). Neurogenin 1 (Ngn1), the bHLH (basic helix–loop–helix) transcription factor, is expressed in the precursors of motor neurons, interneurons and sensory neurons of zebrafish (Blader *et al.*, 1997; Korzh *et al.*, 1998). NeuroD, another bHLH transcription factor, and a downstream target of *Ngn1*, is expressed mostly in postmitotic neurons (Korzh *et al.*, 1998; Mueller and Wullimann, 2002) and is required for terminal differentiation rather than for neuronal precursor commitment. Notch mutant mice up-regulate expression of proneuronal transcription factors (Ngn1 and NeuroD), indicative of premature and excess neuronal development (de la Pompa *et al.*, 1997; Ishibashi *et al.*, 1995) while expression of constitutively active forms of Notch block neuronal development and seemingly maintain neural cells in a precursor state (Gaiano *et al.*, 2000). These observations reinforced the notion that Notch determines neuronal cell fate through lateral inhibition (reviewed in Gaiano and Fishell, 2002).

The homeobox transcription factor hb9 is expressed selectively in postmitotic motor neurons in developing vertebrates and serves as a marker for the motor neuron phenotype (Arber *et al.*, 1999; Tanabe *et al.*, 1998). Genetic studies in mice have shown its role in the

consolidation and maintenance of motor neuron identity (Arber *et al.*, 1999; Thaler *et al.*, 1999). In the *hb9:GFP* fish, *hb9* promoter-driven GFP expression can be monitored in the zebrafish embryos and larvae *in vivo*. In this work, taking advantage of the established transgenic zebrafish line (*hb9-GFP)*, in which, owing to motor neuron-specific expression of GFP, only motor neuron development in the embryos can be monitored *in vivo*, we show that ketamine adversely affects motor neuron development and motor axons as assessed *in vivo* using a transgenic zebrafish line and the resulting phenotype correlates well with the changes in expression of relevant genes.

MATERIALS AND METHODS

Animals

Adult *hb9-GFP* transgenic 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 (IACUC-approved protocol no. E0738701) containing buffered water (pH 7.2) at 28.5 °C, and were fed daily live brine shrimp and Zeigler dried flake food (Zeigler, Gardners, PA, USA). The day–night cycle was maintained at 14:10 h, and spawning and fertilization were stimulated by the onset of light. Fertilized zebrafish embryos were collected from the bottom of the tank. 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 seat at 28.5 °C.

Treatment of Zebrafish Embryos with Ketamine

For each experiment, three sets of 28 hpf dechorionated (manual dechorionation using a pair of watchmakers' forceps) embryos were used. Each set included 10 embryos placed in individual wells of six-well plates (n = 30/each experimental group), each well containing 5 ml egg water. Ketamine (ketamine hydrochloride from Sigma, St Louis, MO, USA; catalog no. K2753) was dissolved as a stock of 100 mg ml^{-1} in buffered egg water. The solution was made fresh and treatment (static exposure) at various doses (0.5 and 2.0 mM) continued for 2 or 20 h. Ketamine stock solution was added to the embryos in 5 ml of egg water to a final concentration of either 0.5 or 2.0 mM ketamine. After 2 h of ketamine treatment, the embryos were examined microscopically (epifluorescence using the FITC filter). Since there was no difference in GFP fluorescence levels in the treated and control embryos, eventually, 20 h static exposure was chosen as there were no changes in the intensities of GFP expression after 2 h treatments, although locomotory behavior changes in zebrafish larvae have been observed after minutes of ketamine treatment (Burgess and Granato, 2007). The rationale behind this longer exposure was to make any subtle effects of ketamine on the phenotype microscopically detectable. An untreated control group of 10 embryos/set (n = 30) was examined in parallel. Embryos were incubated at 28.5 °C.

Live Embryo Morphological Assessment

Morphological changes were examined by visually monitoring (using a Nikon SMZ1000 binocular microscope) the following endpoints: body length, contour and curvature, head

morphology and yolk morphology (color of yolk sac and shape of yolk extension). Ten embryos from each experimental group were examined for these parameters.

Live Embryo Imaging

Post-treatment with ketamine, images of the *hb9-GFP* Tg embryos were acquired using an Olympus SZX 16 binocular microscope and DP72 camera. Higher magnification images were acquired using a Nikon Eclipse 80i microscope and Nikon DXM1200C digital camera. After 2 h of ketamine treatment, when the embryos were 30 hpf, GFP expression in the motor neurons was monitored under the microscope for changes in GFP expression. In embryos treated with ketamine for 20 h (static exposure), GFP-expressing spinal motor neurons in the trunk region (three hemisegments following the distal end of the yolk extension) were counted per specific hemisegments following a procedure used earlier (Kanungo *et al.*, 2009). The values from 10 embryos each per experimental group were averaged to obtain the number of neurons/hemisegment. Relative motor axon (GFP-positive) lengths were measured using a micrometer.

RNA Extraction and cDNA Synthesis

Total RNA (from pooled 30 embryos/treatment group) was extracted from whole embryos (48 hpf) 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) to determine RNA quality (A_{260}/A_{280} >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

The following primers were used for the quantitative polymerase chain reaction (qPCR) assays: Gli2a forward 5′- AAAAACAGGGCGGGACTACT-3′ and reverse 5′-ATGCTGGGTTGGAGGTACAG-3′; Gli2b forward 5′-TTTGCTGGAGCCAGAAAGTT-3′ andreverse5′-TTCGCTGAAGACGTTTCCTT-3′; Notch1a forward 5′-TTCTGGCATTCACTGTGAGC-3′ and reverse 5′-TCTCTCTGTCCTGGCAGGTT-3′; Ngn1 forward 5′-AAG-CAGGGCAAGTCAAGAGA-3′ and reverse 5′-ACGTCGGTTTGCAAGTATCC-3′; NeuroD forward 5′-CAGCAAGTGCTTCCTTTTCC-3′ and reverse 5′-TAAGGGGTCCGTCAAATGAG-3′; GAPDH forward 5′- GATACACGGAGCACCAGGTT-3′ and reverse 5′-GCCATCAGGTCACATACACG-3′.

Real-time PCR (qPCR)

Real-time PCR was performed using a CFX96 C1000 (Bio-Rad, Hercules, CA, USA) detection system with SYBR green fluorescent label (Bio-Rad). Samples (25 ml final volume) 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 $2-\Delta\Delta C_t$ 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 pools of embryos ($n = 30$, from triplicate wells containing 10 per well) were averaged and shown as normalized gene expression ± SEM. One-way ANOVA ('exposure' or 'ketamine dose' as factor) and Holm–Sidak pair-wise multiple comparison *post-hocs* (Sigma Stat 3.1 for analysis) were used to determine statistical significance with $P < 0.05$.

RESULTS

Motor Neuron-specific GFP (Reporter) Expression in Transgenic Zebrafish Exposed to Ketamine

Based on reported observations that ketamine is neurotoxic in developing rodents and monkeys, 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 order to assess the effect of ketamine on the developing zebrafish nervous system *in vivo*, we employed a transgenic zebrafish line (*hb9:GFP*) that has motor neurons specifically identified by the expression of GFP. Based on prior doses of ketamine used with zebrafish larvae for behavioral studies (Burgess and Granato, 2007), we chose two different doses, 0.5 and 2.0 mM, that were directly added to the water. Embryos (28 hpf) exposed for 2 h to ketamine showed no effect on motor neurons when monitored *in vivo* (data not shown). A prolonged exposure to ketamine for 20 h, however, resulted in a reduction in the GFP expression intensity in the motor neuron population at the 2.0 mM dose, but the defects were not so obvious at the lower 0.5 mM dose (Fig. 1). Although no obvious morphological (body contour and curvature, head morphology, yolk sac color and contour of yolk extension) abnormalities were noted after ketamine treatment, scanning for GFP expression in live embryos revealed that, when compared with the control untreated embryos (Fig. 1A), 0.5 mM ketamine-treated embryos did not show any differences (Fig. 1B) while the 2.0 mM ketamine-treated embryos showed reduced GFP expression in the brain and spinal cord (Fig. 1 C).

Ketamine's Adverse Effects on the Motor Neurons and Motor Axon Development in Transgenic Zebrafish

In the brain, exact quantification of GFP-positive neurons either in the control or in the adversely affected 2.0 mM ketamine-treated embryos was not possible The qualitative changes indicated a reduced GFP expression intensity in the ketamine-treated brain compared with the control (Fig. 2A, B). A similarly reduced GFP expression in the spinal cord motor neuron population was noticeable in the 2.0 mM ketamine-treated compared with the control (Fig. 2C, D). In these *hb9:GFP* transgenic embryos, the motor axons, projecting from the spinal motor neurons, also express GFP making it possible to visualize them *in vivo*. On comparison between control and 2.0 mM ketamine-treated embryos, a significant reduction in axon length (20%) was obvious in the latter group (Fig. 2E).

Significant Reduction in Spinal Motor Neurons in Transgenic Zebrafish Exposed to Ketamine

Although it was not possible to visualize or count the cranial motor neurons, using fluorescence microscopy, quantification of GFP-positive spinal motor neurons in specific hemisegments in these embryos was accomplished. By visually counting the neurons in the three hemisegments distal to the yolk extension and obtaining the mean value, the average neuron numbers per hemisegment were calculated. Compared with the control, in the 0.5 mM ketamine-treated embryos, there was no difference in the GFP-positive motor neuron numbers (Fig. 3A, B). However, in the 2.0 mM ketamine-treated embryos, there was a significant reduction (30%) in the number of spinal motor neurons compared with the untreated embryos (Fig. 3C, D).

Changes in the Expressions of Specific Genes Involved in Motor Neuron Development upon Ketamine Exposure

The above adverse effects of ketamine indicated that either there was a reduction in the differentiation of the motor neurons or the differentiated neurons simply did not survive, thus contributing toward a reduction in number. In order to further address these possibilities, we examined the expression of a number of specific genes that are known to regulate motor neuron induction and differentiation as well as some that are expressed in differentiated neurons. Of the neurogenic genes, we choose *Notch1a* (Appel and Eisen, 1998; Chitnis and Kintner, 1996; Chitnis, 1995; Dornseifer *et al.*, 1997; Haddon *et al.*, 1998) and of the proneural genes, *Neurogenin1 (Ngn1*) and *NeuroD* (Blader *et al.*, 1997; Kim *et al.*, 1997; Lee, 1997). Of the genes that have specific motor neuron inductive ability essential for neuronal differentiation from undifferentiated cells, a candidate gene is *Gli2b* (Karlstrom *et al.*, 2003; Ke *et al.*, 2005, 2008). We chose to analyze both *Gli2b* and *Gli2a* since *Gli2a* has been shown to be involved in the development of ventral neurons in the developing mouse brain (Blaess *et al.*, 2006).

Quantitative RT-PCR assays to determine mRNA expression levels (Fig. 4) demonstrated that, compared with the control embryos, 0.5 mM ketamine significantly down-regulated *Gli2b* expression (0.33-fold, $P < 0.0001$), even though no adverse effects on the number or GFP-fluorescence of motor neurons were apparent. *Gli2a* expression was unchanged while the expression of the neurogenic gene, *Notch1a*, was slightly down-regulated (0.8-fold), indicating that ketamine at this dose positively affected the signaling pathway responsible for driving cells toward neuronal commitment. In support of this supposition, the pro-neural gene *Ngn1* was up-regulated (1.5-fold, $P < 0.005$). Since *Ngn1* is a downstream target of Notch inhibition, the results are consistent in showing that the neuronal induction pathway associated with ketamine treatment not only remained intact, but was also somehow favored. Surprisingly, however, *NeuroD*, a direct downstream target of *Ngn1*, was significantly down-regulated (0.7-fold, $P < 0.03$) in the 0.5 mM ketamine-treated embryos. It is possible that, as a gene required for terminal neuronal differentiation in differentiated neurons, *NeuroD* expression began to lessen as a secondary effect while the expression of its inducer, *Ngn1*, remained favored, when differentiating/differentiated neurons were entering, subtly, an apoptotic or disintegrative pathway. This subtle insult, however, was not yet translated to reduced GFP expression, as the motor neurons still existed. If this were the case and if such

down-regulation occurred at the threshold level of an insult at which neurons could remain alive, one would expect any further down-regulation of *NeuroD* to be deleterious to the differentiated motor neurons. Anticipating such an outcome, gene expression changes were analyzed in embryos exposed to 2.0 mM ketamine, a 4-fold higher dose. *Gli2b* expression, although significantly less than in controls (0.5-fold, P 0.0004), was higher than in embryos treated with 0.5 mM, suggesting that, perhaps since *Gli2b* is also expressed in undifferentiated neurons and their precursors, there could be enhancement of the development of these cells. *Gli2a* expression remained unaltered as in the embryos treated with the 0.5 mM dose, but *Notch1a* expression was further down-regulated (0.75-fold, $P <$ 0.005) from levels seen in embryos treated with the 0.5 mM dose. Accordingly, *Ngn1* expression remained up-regulated (1.24-fold, $P < 0.02$). These results further support the hypothesis that ketamine favored the expression of the pro-neural gene, *Ngn1*, by antagonizing Notch signaling. It is important to note that ketamine, at both doses tested, had negative effects on the expression of the neurogenic gene, *NeuroD*, and the motor neuron inductive gene, *Gli2b*. At 2.0 mM, *NeuroD* was down-regulated (0.45-fold, $P < 0.006$). With respect to the *NeuroD* expression pattern, phenotypic comparisons between the 0.5 and 2.0 mM exposure groups suggested that, at the higher dose, the down-regulation of *NeuroD* expression could have reached a threshold level so as to induce neuronal degeneration. The expression pattern of these selected neuron-specific and motor-neuron specific genes that accompanies ketamine treatment compares well with the observed phenotype where motor neurons are adversely affected. At the same time, these results also demonstrate that ketamine does not seem to have any deleterious effects on neuronal commitment, an event that occurs early in neurogenesis, since *Notch1a* was inhibited (a process that promotes neuronal commitment of progenitor cells) and, as would be expected of its downstream effector, *Ngn1* expression was induced. Together, the phenotypic analyses and the specific gene expression studies suggest that initiation of ketamine's deleterious effects on the motor neurons probably begins at the lower 0.5 mM dose, becoming more obvious at the higher 2.0 mM dose, a scenario that probably depends upon the real exposure (internal dose) of the embryos to ketamine.

DISCUSSION

Ketamine is a dissociative anesthetic introduced in the 1960s that produces anesthesia, analgesia, suppression of fear and anxiety, and amnesia. Most of ketamine's effects are mediated by antagonism of NMDA receptors (Anis *et al.*, 1983). Activation of NMDARs is essential for long-term potentiation and spatial learning and memory (Malenka and Bear, 2004), and NMDAR blockade results in impaired synaptic plasticity manifested in adverse effects on learning and memory (Sakimura *et al.*, 1995; Shimizu *et al.*, 2000). Conversely, enhanced NMDAR function improves memory (Tang *et al.*, 2001). It has been shown that higher doses of ketamine can induce apoptosis in rodents (Ikonomidou *et al.*, 1999; Lahti *et al.*, 2001; Larsen *et al.*, 1998; Malhotra *et al.*, 1997; Maxwell *et al.*, 2006; Wang *et al.*, 2006) and primates (Haberny *et al.*, 2002; Slikker *et al.*, 2007; Wang *et al.*, 2006) during the brain growth spurt (Slikker *et al.*, 2007).

Results from the present study on the effects of ketamine on zebrafish embryos provide evidence that, after 20 h of exposure, 2.0 mM ketamine significantly reduces both cranial

and spinal motor neuron populations. These data are concordant with reported data from mammalian studies (neurotoxicity induced by ketamine in rodents and monkeys). It has been shown that, in postnatal day 7 rat pups, repeated doses of 20 mg kg^{-1} ketamine resulted in a blood level of 14 μg ml^{-1} concentration and induced neuroaopotosis in the dorsolateral thalamus (Scallet *et al.*, 2004). The blood level of 14 μg ml^{-1} is approximately 7-fold greater than anesthetic blood levels in humans (Malinovsky *et al.*, 1996; Mueller and Hunt, 1998). The doses we used, 0.5 (1.37 mg ml^{-1} water) and 2.0 mM (5.48 mg ml^{-1} water) are in the range of the effective doses that induce behavioral changes in zebrafish larvae (Burgess and Granato, 2007). In the present study, the focus was specifically on motor neurons as we took advantage of the transgenic line that makes motor neurons visible *in vivo*. Ketamine induced motor neuron toxicity *in vivo* as assessed using *hb9-GFP* transgenic embryos. There was also a significant reduction in spinal motor axon length, indicating that either ketamine had a direct effect on the axonogenesis or the exposed neurons were producing stunted axons.

In order to gain more insight into the occurrence of such phenotypes, the expression of selected target genes that are associated with motor neuron development and axonogenesis was analyzed. Gene expression profiling in the developing rat brain exposed to ketamine has revealed the up-regulation of several NMDA receptor subunits (Shi *et al.*, 2010). In zebrafish, ketamine treatment began at 28 hpf, when only NR1.1 and NR 2D.1 are found in brain and spinal cord. NR1.2 is expressed in the brain at 48 hpf but not in the spinal cord (Cox *et al.*, 2005), a time point when ketamine exposure was terminated. In this initial study, these time points were chosen since the goal was to monitor ketamine's effects *in vivo* and, beyond 48 hpf, excess pigmentation hinders visualization of GFP signals. Although development of zebrafish pigmentation can be inhibited by early treatment of embryos with phenylthiourea to facilitate visualization of tissues and organs and, therefore, GFP signals, phenylthiourea is a known toxic compound and was not used in these experiments. Our immediate goal was to focus on a set of specific genes, *Gli2a*, *Gli2b*, *Notch1a*, *Ngn1* and *NeuroD* that are essential for motor neuron development and axonogenesis.

During neurodevelopment in vertebrates, the formation of oligodendrocytes and motor neurons depends on hedgehog (Hh), a family of secretory proteins, and Glis, the zinc finger proteins that act as mediators of Hh signaling (Ruiz i Altaba, 1999). Mice lacking sonic hedgehog (Shh) function display severe neural patterning defects, including a lack of most ventral neuronal cell types in the spinal cord (Chiang *et al.*, 1996). It has also been suggested that Shh signaling is required to promote the timely appearance of motor neuron progenitors in the mouse developing spinal cord (Oh *et al.*, 2009). In mouse embryos lacking Gli genes, the ventral telencephalon is highly disorganized with intermingling of different neuronal cell types (Yu *et al.*, 2009). $Gli2^{-/-}$; $Gli3^{-/-}$double mutant mice have an enlarged ventral neuroepithelium resulting from enhanced cell proliferation (Bai *et al.*, 2004). There are two *Gli2* genes, *Gli2a* and *Gli2b*, in zebrafish (Ke *et al.*, 2005) that play a role in the development of several cellular lineages, such as the ventral neural precursors, cranial motor neurons, interneurons and dorsal sensory neurons (Ke *et al.*, 2008). *Gli2a* is preferentially expressed early on in the lateral mesoderm. *Gli2b* is also expressed early on, predominantly in the neural plate (Karlstrom *et al.*, 2003), and its absence causes defects in the neural tube, including the reduction of mitotic neural precursors (Karlstrom *et al.*, 2003; Ke *et al.*, 2005). In ketamine-treated zebrafish embryos, *Gli2a* expression remained unaltered while that of

Gli2b was down-regulated. Since *Gli2b* but not *Gli2a* is directly involved in motor neuron development, ketamine-induced motor neuron loss could be the result of such a down-regulation. In our studies, stunted axonogenesis in ketamine-treated embryos is consistent with the report that loss of *Gli2b* function causes abnormal axonogenesis (Ke *et al.*, 2008). A possible mechanism of how *Gli2b* down-regulation could influence motor neuron toxicity is schematically presented in Fig. 5.

Inhibition of Notch signaling results in the formation of excess primary neurons whereas forced expression of constitutively active Notch blocks neuronal differentiation (Atkinson and Roy, 1995; Chitnis, 1995; Coffman *et al.*, 1993). During neuronal differentiation, pro-neural genes, such as *Ngn1*, a direct downstream target of Notch inhibition, induce downstream bHLH genes, such as *NeuroD*, to elicit the transition from proliferative neural precursor cells to postmitotic neurons (Blader *et al.*, 1997; Kim *et al.*, 1997; Lee, 1997). Our results demonstrated that ketamine induced a down-regulation of *Notch1a* with an up-regulation of *Ngn1* expression, an occurrence that favors neuronal precursor commitment. What could then be deleterious to the motor neurons may be the down-regulation of *NeuroD*, which is the only gene that showed a dose-dependent response to ketamine, thus explaining a scenario where only the higher dose resulted in a significant loss of motor neurons. *NeuroD* is a direct downstream target of *Ngn1* that is expressed in differentiated neurons and is essential for terminal differentiation of neurons (Korzh *et al.*, 1998; Mueller and Wullimann, 2002). In our studies, while *Ngn1* was up-regulated, surprisingly, *NeuroD* was down-regulated. One possible explanation of this observation is that, since ketamine induced *Notch1a* down-regulation and Notch signaling is necessary for neuron survival (Breunig *et al.*, 2007; Mason *et al.*, 2006; Oishi *et al.*, 2004), a sustained Notch inhibition in differentiated neurons may have triggered motor neuron degeneration, although the same Notch inhibition in the proneural domain is required for the generation of neuronal progenitors (Fig. 5). Since *NeuroD* is expressed in differentiated neurons but not in their progenitors, its low expression may be a consequence of fewer living neurons post ketamine treatment.

Taken together, our *in vivo* data suggest that ketamine is toxic to motor neuron development in zebrafish. Phenotype-specific selected gene expression studies indicate that ketamine could potentially favor neuronal commitment but negatively affect neuronal differentiation and survival.

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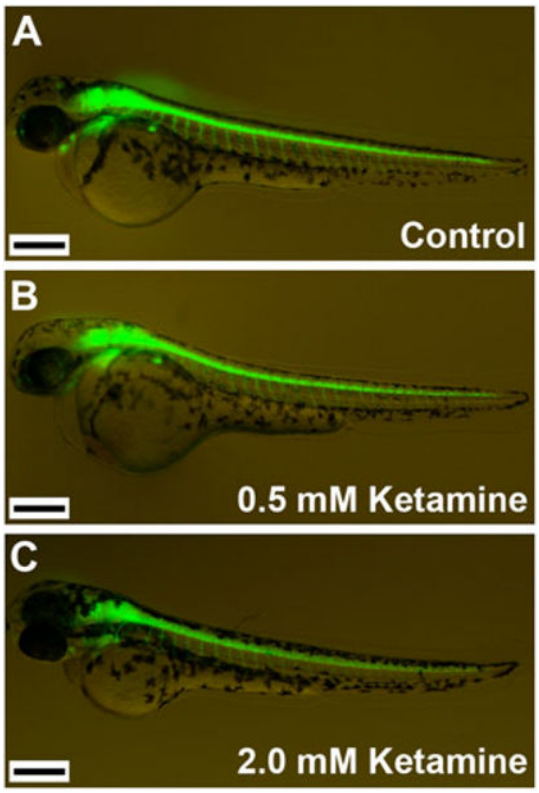

Figure 1.

Effect of ketamine on zebrafish embryos. Ketamine does not have a drastic effect on zebrafish morphology but adversely affects the central nervous system as indicated by differences in relative GFP fluorescence. In the *hb9:GFP* transgenic fish motor neurons express *hb9* promoter-driven GFP. Embryos at 28 hpf were treated with ketamine. After 20 h of treatment (48 hpf actual age), images of the live embryos were acquired for assessment of GFP fluorescence. In this experiment, MS-222 was not used (a routine procedure) to immobilize the untreated control embryos for photography in order to avoid any interference with the effect produced by ketamine. The experiment was repeated three times with $n = 30$ (replicates of 10 each) for each group in each experiment. Lateral views of the embryos are shown with dorsal side up. Embryos in different experimental groups are (A) control, (B) 0.5 mM ketamine-treated, and (C) 2.0 mM ketamine-treated. Scale bar = 280 μM.

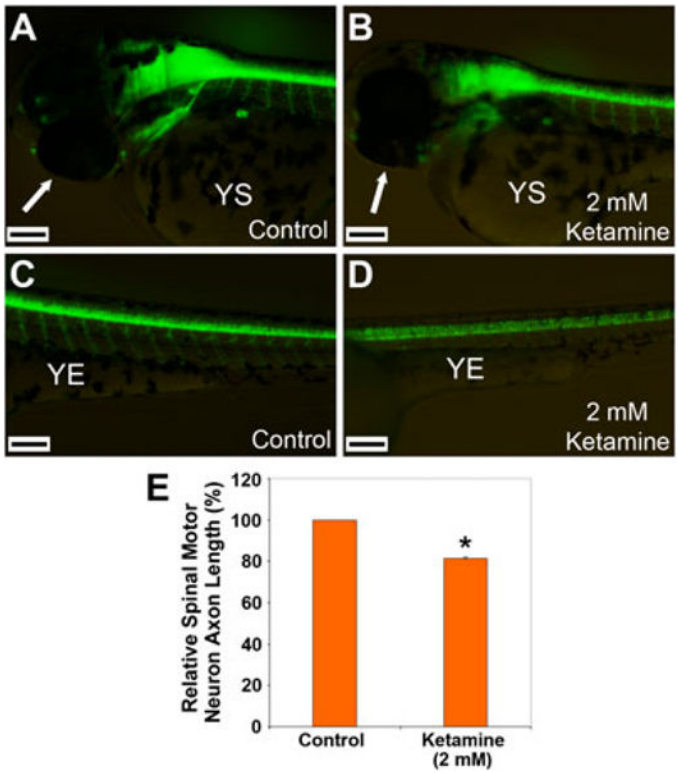

Figure 2.

Adverse effects of ketamine on cranial and spinal motor neurons. *Hb9:GFP* transgenic fish embryos (28 hpf) were treated with 2.0 mM ketamine. After 20 h of treatment (48 hpf actual age), images of the live embryos (lateral views with dorsal side up and anterior side to the left) were acquired for assessment of GFP fluorescence. GFP expressing motor neurons are shown in control (A) and ketamine-treated (B) brains, and control (C) and ketamine-treated (D) spinal cords. The arrows indicate the eyes, YS indicates yolk sac, and YE indicates yolk extension. Since the brain has a high density of GFP-positive motor neurons, the difference in motor neuron numbers could not be quantitatively determined. However, overall GFP fluorescence in ketamine-treated embryos was reduced compared with the untreated controls. Spinal motor neurons, however, could be visualized individually. Scale bar = 120 μM. Axon lengths from specific areas in the spinal cord region were measured using a micrometer in the microscope and the mean difference (%) between the control and ketamine-treated embryos from three different replications is presented (E). The value for the control does not have an error bar (SEM) because the spinal motor axon lengths were normalized relative to the lengths of the control. Student's *t*-test was performed to determine statistical significance between observed differences and significance (*) was set at $P < 0.05$ (D).

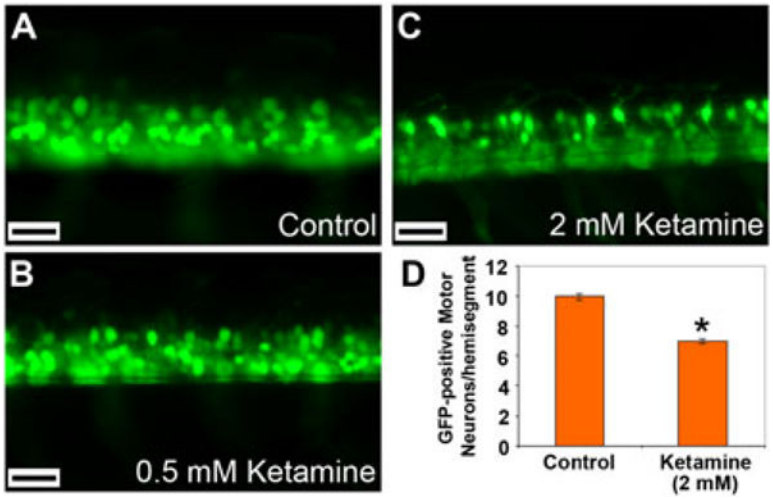

Figure 3.

Effect of ketamine on spinal motor neuron numbers. Embryos at 28 hpf were treated with 0.5 and 2.0 mM ketamine for 20 h (static exposure). Higher magnification of the trunk region showing GFP positive neurons in control (A), 0.5 mM ketamine treated- (B) and 2.0 mM ketamine-treated (C) embryos (48 hpf actual age). GFP-positive motor neurons in specific hemi-segments were counted, and Student's *t*-test was performed to determine statistical significance between observed differences with significance (*) set at $P < 0.05$ (D). In each of three replications, spinal motor neurons were counted in 10 embryos each from the control and ketamine-treated groups. Scale bar = 30 μM.

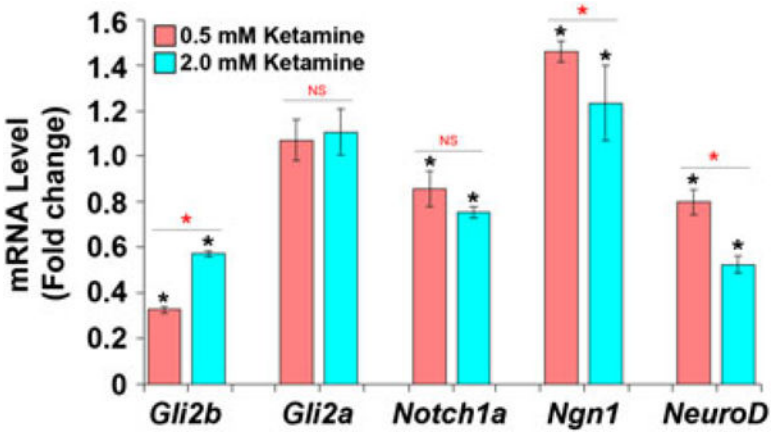

Figure 4.

Effect of ketamine on expression of specific proneural and neurogenic genes. Ketamine induces changes in gene expression in zebrafish embryos. Expression of the proneural gene *Notch1a*, and the neurogenic genes *Ngn1*, *NeuroD*, *Gli2a* and *Gli2b* at the mRNA level was analyzed. Embryos at 28 hpf were treated with 0.5 and 2.0 mM ketamine. After 20 h of treatment (48 hpf actual age), total RNA was isolated from control (untreated) and the two ketamine-treated groups. Following first-strand cDNA synthesis from the RNA, qPCR was performed. The $2-\Delta\Delta C_t$ method was used to determine the relative gene expression. The GAPDH gene was the internal control for all qPCR experiments. The mean C_t values for GAPDH expression were 21.47 ± 0.11 (control), 21.28 ± 0.07 (0.5 mM ketamine) and 21.02 ± 0.073 (2 mM ketamine). Data from biological replicates were averaged and are shown as normalized gene expression ± SEM. For all pairwise multiple comparison procedures the Holm–Sidak method was used for data analysis with overall significance level set at $P < 0.05$. Lower-level ANOVAs were performed to determine further differences (between different ketamine doses).

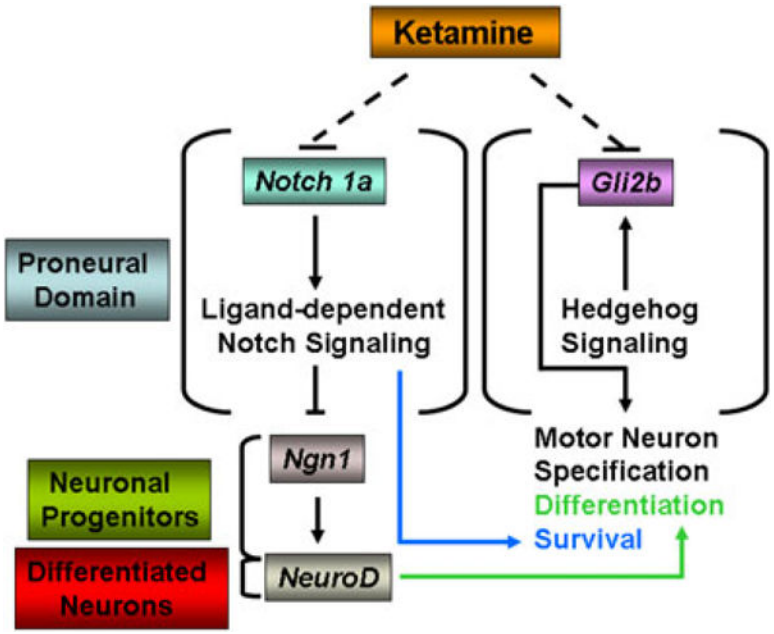

Figure 5.

Schematic representation of pathways potentially affected by ketamine. Ketamine has toxic effects on motor neurons in the zebrafish embryos. Down-regulation of the transmembrane receptor gene, *Notch1a*, could negatively affect ligand-dependent Notch signaling and inhibition of Notch signaling in the proneural domain is known to up-regulate the neurogenic gene, *Ngn1*, in the neuronal progenitors. *Ngn1* is a direct inducer of *NeuroD* that is critical for neuronal differentiation and *NeuroD* is expressed in differentiated neurons. At the same time, in differentiated neurons, sustained inhibition of Notch signaling can adversely affect neuron survival. Ketamine-induced down-regulation of *Gli2b*, a mediator of Hedgehog signaling, can disrupt Hedgehog signaling, thereby adversely affecting motor neuron specification,differentiation and survival. Down-regulation of *NeuroD* expression in spite of an up-regulation of its inducer, *Ngn1*, could be the consequence of fewer surviving differentiated neurons.