

Potential Neurotoxicity of Ketamine in the Developing Rat Brain

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Ketamine, an N-methyl-D-aspartate (NMDA) receptor ion channel blocker, is a widely used anesthetic recently reported to enhance neuronal death in developing rodents and nonhuman primates. This study evaluated dose-response and time-course effects of ketamine, levels of ketamine in plasma and brain, and the relationship between altered NMDA receptor expression and ketamine-induced neuronal cell death during development. Post-natal day 7 rats were administered 5, 10, or 20 mg/kg ketamine using single or multiple injections (subcutaneously) at 2-h intervals, and the potential neurotoxic effects were examined 6 h after the last injection. No significant neurotoxic effects were detected in layers II or III of the frontal cortex of rats administered one, three, or six injections of 5 or 10 mg/kg ketamine. However, in rats administered six injections of 20 mg/kg ketamine, a significant increase in the number of caspase-3- and Fluoro-Jade C-positive neuronal cells was observed in the frontal cortex. Electron microscopic observations showed typical nuclear condensation and fragmentation indicating enhanced apoptotic characteristics. Increased cell death was also apparent in other brain regions. In addition, apoptosis occurred after plasma and brain levels of ketamine had returned to baseline levels. *In situ* hybridization also showed a remarkable increase in mRNA signals for the NMDA NR1 subunit in the frontal cortex. These data demonstrate that ketamine administration results in a dose-related and exposure-time dependent increase in neuronal cell death during development. Ketamine-induced cell death appears to be apoptotic in nature and closely associated with enhanced NMDA receptor subunit mRNA expression.

Key Words: N-methyl-D-aspartate (NMDA) receptor; neuronal cell death; apoptosis.

Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, produces a dose-related state of unconsciousness and analgesia commonly referred to as dissociative anesthesia (Kohrs and Durieux, 1998). Clinically, ketamine's role in

pediatric anesthesia is well established. However, recent studies have found that ketamine may cause dose-dependent widespread apoptotic neurodegeneration in the immature rat brain (Ikonomidou *et al.*, 1999; Jevtovic-Todorovic *et al.*, 2003; Scallet *et al.*, 2004). As a result, the possible toxic effects of ketamine on the immature brain are being extensively explored. It has been hypothesized that the developing nervous system may be more susceptible than the mature brain to some neurotoxic insults. During development in the rat, the window of vulnerability to the toxic effects of ketamine is restricted to the period of rapid synaptogenesis, also known as the brain growth spurt, which occurs immediately after neurons have differentiated and migrated to their final destinations. It is postulated that, in rodents, excessive suppression of neuronal activity by ketamine during the brain growth spurt triggers neurons to commit "suicide" via apoptosis (Ikonomidou *et al.*, 1999; Jevtovic-Todorovic *et al.*, 2003; Olney *et al.*, 2002).

The administration of noncompetitive NMDA receptor antagonists, such as ketamine, phencyclidine (PCP), and MK-801, to rats during a critical period of development results in neurotoxicity/neurodegeneration in several major brain areas (Ikonomidou *et al.*, 1999; Scallet *et al.*, 2004). Ketamine has long been known to be a noncompetitive NMDA receptor ion channel blocker. Neurotoxicity in postnatal day 7 (PND-7) rats, presumably due to the dysregulation of NMDA receptors, has been proposed to be an important mechanism of apoptotic neurodegeneration induced by ketamine (Slikker *et al.*, 2007; Wang *et al.*, 2005, 2006). Several lines of evidence have indicated a close relationship between the blockade of NMDA receptors and neurodegeneration, but the underlying mechanism involved in such effects remains unknown. It has been postulated that the continuous exposure of developing brains to ketamine causes selective cell death by a mechanism that involves a compensatory upregulation of NMDA receptor subunits (Wang *et al.*, 2005, 2006). This upregulation initiates a cascade beginning with Ca^{2+} influx which produces an increase in reactive oxygen species (ROS) that appears to originate in the mitochondria (Johnson *et al.*, 1998; Slikker *et al.*, 2005). This Ca^{2+} loading beyond the buffering capacity

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of the mitochondria reduces the membrane potential and disrupts electron transport, leading to increased production of ROS (Slikker *et al.*, 2005; Wang *et al.*, 2000). A major emphasis of this study was to determine the robustness of ketamine-induced neurotoxicity in rat brain by examining neuropathological and neurobiological outcomes. It is proposed that the administration of ketamine during critical developmental periods will result in a dose-related and exposure-time dependent increase in neuronal loss. NMDA receptor dysfunction may be a key mechanism underlying the enhanced apoptosis induced by ketamine.

METHODS

Ketamine administration. Seven-day-old (PND-7) Sprague Dawley (male and female) rat pups (average body weight, 12–18 g) were used to examine the potential neurotoxic effects of ketamine. All animal procedures were approved by the Institutional Animal Care and Use Committee of the National Center for Toxicological Research, and conducted in full accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Ketamine hydrochloride (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) was diluted in saline, and the identity and purity confirmed with high-performance liquid chromatography (HPLC)/mass spectrometry (MS). Rat pups were randomly assigned to one of nine dose groups and to either the histology or blood level experiment. Pups were dosed subcutaneously with either ketamine hydrochloride or saline (10 μ l/g) using a 30-gauge needle, and were returned to their dam between injections to provide warmth and reduce potential stressors. Doses of ketamine (5, 10, or 20 mg/kg, respectively) were administered in one, three, or six injections, respectively, at 2-h intervals. Control animals received saline at the same time points. In all cases, a 6-h withdrawal period was allowed before animals were sacrificed via transaortic perfusion of 0.9% saline and paraformaldehyde (4%) in 0.1M phosphate buffer (pH 7.2) for the histochemical studies. For each dose and injection time points, rats were randomly assigned to treatment and control groups ($n = 5$ per group).

Measurement of ketamine in rat brain and plasma. Rats receiving six doses of 20 mg/kg ketamine or saline were sacrificed by decapitation at 5 min, 1, 2, 4, 6, and 18 h following the last injection. Blood and brain tissue (frontal cortex) were collected for analyses of ketamine and norketamine. Plasma was immediately isolated from whole blood by centrifugation at $1046 \times g$ for 10 min at 4°C. The plasma supernatant was transferred to plastic cryovials and stored at –70°C until assayed. For measuring brain ketamine and norketamine levels, coronal brain sections (0.15 g; 1 mm thickness) including frontal cortex were cut corresponding to Figure 79 as defined by the Atlas of the Developing Rat Brain (Paxinos *et al.*, 1991). Brain tissue was homogenized in 0.5 ml phosphate buffer (0.1M; pH 7.4) and stored at –70°C until assayed. Concentrations of racemic ketamine and norketamine were quantified in plasma and brain tissue using a validated method based on HPLC/MS/MS with isotope dilution, as previously reported (Slikker *et al.*, 2007). When using 10- μ l plasma samples or 15-mg brain tissue equivalents, the limits of quantitation for both ketamine and norketamine were approximately 1.2 ng/ml for plasma (0.005) and 0.8 ng/g for brain tissue, respectively. As previously reported, the interday and intraday precision for these measurements were in the range of 2–12% and accuracies in the range of 102–114% (Slikker *et al.*, 2007). For quality control purposes, blank plasma samples, ketamine/norketamine spiked plasma samples, and a mixed standard containing all labeled and unlabeled analytes were run with every sample set. Model-independent pharmacokinetic analysis was performed using PK solutions 2.0 software (Summit Research Solutions, Montrose, CO).

Cell death detection ELISA. Apoptosis was studied by measuring the cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) by photometric enzyme-immunoassay (cell death detection enzyme-linked

immunosorbent assay [ELISA]; Roche Diagnostics Corporation, Indianapolis, IN). Coronal brain sections (1 mm thickness) including frontal cortex were taken for cell death detection ELISA assay. Briefly, brain tissue was homogenized in 3 ml of lysis buffer (provided by manufacturer) and incubated for 30 min at room temperature. After centrifugation to remove nuclei and cellular debris, the supernatants (cytosol containing low-molecular mass, fragmented DNA) were diluted 1:2 (vol/vol) with lysis buffer. Then, 20 μ l from each sample were transferred to a 96-well plate precoated with antihistone antibody to which 80 μ l immunoreagent mix were added. After incubation and washes, the wells were treated with the chromogen 2,2'-azinobis (3-ethylbenzthiazoline) sulfonic acid as a substrate. The intensity of the color that developed was measured at 405 nm, whereas that at 490 nm was used as a blank (reference wavelength). Rat pups were randomly assigned to treatment and control groups ($n = 5$ per group).

Data were analyzed using parametric analysis of variance (ANOVA), and Tukey's *post hoc* analysis was used to correct for multiple comparisons when applicable. One-way ANOVA was used and the null hypothesis was rejected at a probability level of $p < 0.05$.

Electron microscopy. Electron microscopy (EM) was used to verify the nature of the ketamine-induced neurodegeneration. All rats ($n = 5$ per group; control and ketamine-treated, 20 mg/kg \times 6 injections) were sacrificed via transaortic perfusion of 0.9% saline and 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), then the brain (frontal cortex) was removed and kept in the same fixative. The frontal cortex was postfixed with 1% osmium tetroxide in 0.1M cacodylate buffer, and subsequently washed with 25% and 50% ethanol plus 5% uranyl acetate, followed by dehydration in an ascending ethanol series and embedded in Epon. The frontal cortex semithin sections (1 μ m) were counterstained with Toluidine Blue dye and examined under a Nikon light microscope. The thin sections were stained with uranyl acetate and lead citrate, and examined at 60 kV using a Philips CM100 electron microscope (FEI Corporation, Eindhoven, The Netherlands).

Immunocytochemical study for activated caspase-3. All rat pups in the histopathological studies were perfused with saline, and then fixed by transaortic perfusion of ice-cold (4°C) paraformaldehyde (4%) in phosphate buffer (pH 7.2) 6 h after ketamine administration. The rat pup brains were then equilibrated with sucrose and embedded in optimal cutting temperature tissue medium. In order to measure the density and distribution of caspase-3 and Fluoro-Jade C–positive neural cells, the following procedure was followed: The whole rat brain including frontal cortex (corresponding to Figure 79 as defined by the Atlas of the Developing Rat Brain; Paxinos *et al.*, 1991), striatum (corresponding to Figure 81 of the Atlas), hippocampus (corresponding to Figure 93 of the Atlas), thalamus (including dorsolateral thalamus) (corresponding to Figure 97 of the Atlas), and amygdala (corresponding to Figure 99 of the Atlas) were examined and adjacent pre- and post-serial sections (for every brain region) were selected for morphological assessments.

Coronal cryostat sections (10 μ m) through the whole brain were processed by procedures described previously (Muller *et al.*, 1996), using a primary anti-active caspase-3 antiserum raised in rabbits (Trevigen, Gaithersburg, MD). Briefly, sections were first washed in phosphate-buffered saline (PBS) for 1 h, permeabilized in PBS/0.5% bovine serum albumin (BSA)/0.03% triton for 30 min at room temperature and incubated with the primary antibodies at 4°C overnight. A rabbit polyclonal antibody to caspase-3 (1:200) was used to identify activated caspase-3, one of the key effectors of apoptosis. Bound antibodies were revealed with horseradish peroxidase–conjugated sheep anti-rabbit secondary antibodies (diluted 1:100, Roche, in PBS/0.5% BSA) for 2 h. After a series washes in PBS, the reaction complexes were developed by incubating the sections in 0.02% 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO). The control sections were processed identically and in parallel; however, they were incubated with PBS/BSA solution instead of the primary antibodies. The immunostaining was examined with an Olympus light microscope (Olympus America Inc., Center Valley, PA).

Fluoro-Jade C staining. As described previously (Schmued *et al.*, 2005), adjacent sections (10 μ m) were mounted onto subgelatinized slides. Slides were first immersed in a basic alcohol solution consisting of 1% NaOH in 80% ethanol for 5 min, followed by a wash for 2 min in 70% ethanol. The tissue was

briefly rinsed with water and incubated in 0.06% KMnO_4 solution for 10 min. Slides were then transferred for 10 min to a 0.0001% solution of Fluoro-Jade C (Histo-Chem, Inc. Jefferson, AR) dissolved in 0.1% acetic acid. The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid vehicle. The slides were rinsed through three changes of distilled water for 1 min per change. The air-dried slides were cleared in xylene and then coverslipped with DPX nonfluorescent mounting media (Sigma). The Fluoro-Jade C staining was examined with an Olympus light microscope equipped with epifluorescence.

Quantitative analysis. Unbiased sampling of brain sections was performed by randomly selecting five viewing fields ($10\times$) per section from five serial sections of frontal cortex, striatum, hippocampus, thalamus and amygdala individually, for each brain. To determine the degree of neurodegeneration in a given brain region, a PC-based Image Analysis System (MCID [Microcomputer Imaging Device], Imaging Research, Inc., St Catherines, Ontario, Canada) interfaced to an Olympus Vanox microscope via a solid state video camera was used for image analysis. The viewing fields (photographs) were counted by a trained expert and later confirmed by two raters blind to the treatment. Differences between control and ketamine-treated animals with three dose levels were statistically evaluated by one-way ANOVA and Tukey's post hoc analysis was used to correct for multiple comparisons when applicable.

In situ hybridization. An oligonucleotide probe complementary to the mRNA encoding the NMDA receptor NR1 subunit was selected on the basis of cloned cDNA sequences. The sequence of the probe used for *in situ* hybridization was as follows: 5'-TTCCTCCTCCTCCTC-CTGTTACACCTT-GAATCGGC-CAAAGGGACT (this corresponds to a region that is constant across all NR1 splice variants). It was 3' end-labeled by incubation with [^{35}S]deoxy-ATP (New England Nuclear, Boston, MA) and terminal deoxy-nucleotidyl transferase (Boehringer Mannheim Corp., Indianapolis, IN) to attain specific activities of approximately $5\text{--}8 \times 10$ cpm/ μg . The specificity of the probes has been previously described (Monyer *et al.*, 1992; Moriyoshi *et al.*, 1991).

Coronal sections ($10\ \mu\text{m}$) through the frontal cortex were cut with a cryostat, rinsed in PBS and processed for *in situ* hybridization as described previously (Bartanusz *et al.*, 1993). After an overnight hybridization at 41°C , slides were washed successively in $4\times$, $1\times$, and $0.1\times$ sodium chloride-sodium citrate solution, quickly dehydrated in ethanol (70%), and air-dried. Autoradiography was performed using Kodak (Rochester, NY) NTB3 emulsion; slides were exposed for 3 weeks at 4°C . Analysis of *in situ* hybridization autoradiographs was accomplished on hematoxylin-eosin counterstained sections.

For quantitation of *in situ* autoradiographs, images were acquired with the MCID (Imaging Research, Inc.) for analysis. Briefly, the images were smoothed with a 3×3 square filter to remove noise and regions of interest (ROIs) were selected using a threshold technique that segments the image into labeled cells and background. The threshold was held constant for all ROI within each section. The density of silver grains associated with neurons was estimated by measuring the area within a $20\text{-}\mu\text{m}$ fixed diameter circle placed over individual neurons that exceeded the threshold value. Background labeling was determined in a similar fashion and was subtracted from each measurement to estimate specific labeling in each ROI. A monotonic relationship was assumed to exist between measured labeling and the amount of mRNA labeled with the radioactive probe. This technique is similar to that used by several laboratories except that a fixed size rather than a variable size ROI was used.

RESULTS

Ketamine Levels in Plasma and Brain during the Withdrawal Phases

After multiple ketamine injections to PND-7 rats ($20\text{ mg/kg} \times 6$), plasma ketamine levels ranged from $5.80 \pm 3.10\ \mu\text{g/ml}$ at

5 min to $0.01 \pm 0.01\ \mu\text{g/ml}$ at 18 h (mean \pm SEM) (Fig. 1A). The ketamine levels in brain (wet tissue) ranged from $2.65 \pm 1.60\ \mu\text{g/g}$ at 5 min to $0.03 \pm 0.02\ \mu\text{g/g}$ at 18 h (Fig. 1A). Ketamine levels in plasma were highest at 5 min after ketamine administration, at which time the animals were unconscious. Ketamine levels in brain tissue were lower than in plasma, but similar to plasma, were also highest at 5 min after ketamine administration. Ketamine concentrations in plasma and brain decreased in a manner consistent with a single elimination process with a half-time of 0.68 and 0.90 h, respectively. In addition, the metabolite norketamine peaked at $\sim 6.7\ \mu\text{g/ml}$ (in plasma) and $\sim 3.5\ \mu\text{g/ml}$ (in brain tissue) 2 h after the last ketamine injection, and the norketamine levels

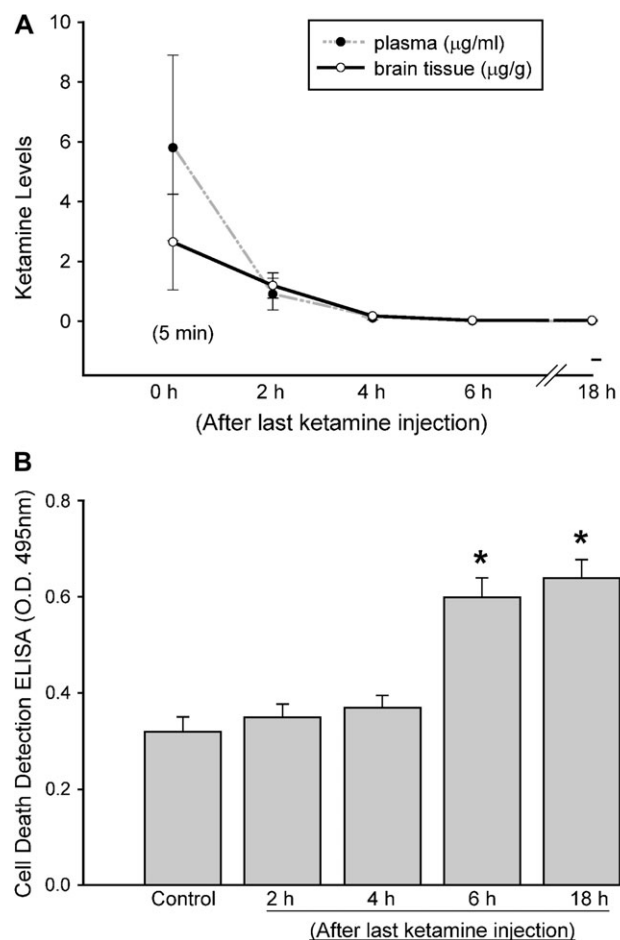


FIG. 1. Ketamine concentrations in plasma or brain and potential neurotoxicity following a 5-min, 2-, 4-, 6-, or 18-h withdrawal period. Ketamine ($20\text{ mg/kg} \times$ six injections) was administered subcutaneously at 2-h intervals ($n = 5$ per group). Ketamine levels, both in plasma and brain, were relatively high after 5 min and then began to sharply decrease by 2–4 h, and reached approximately zero 6 h after ketamine administration (A). Quantitative analysis of ketamine-induced neurotoxicity as indicated by cell death detection ELISA is shown in B. For each condition, at least five animals ($n = 5$ per group) were assayed. The cell death ELISA data are presented as means \pm SEM. A probability of $*p < 0.05$ was considered significant (one-way ANOVA with Holm-Sidak test).

decreased to undetectable levels approximately 6 h after the last ketamine injection (data not shown). Norketamine concentrations in plasma and brain changed in a manner consistent with formation, distribution, and elimination processes with half-times of 0.09, 2.0, and 3.1 h for plasma and 0.08, 1.9, and 6.1 h, respectively.

Assessment of Ketamine-Induced Neurotoxicity

This study sought to define the relationship between plasma and brain ketamine levels and the potential neurotoxicity of ketamine. Potential neurotoxicity in rat brains was initially measured with Cell Death Detection ELISA. After multiple ketamine injections to PND-7 rats ($20 \text{ mg/kg} \times 6$), assessment of the effects of plasma and brain ketamine levels on fragmented DNA, a potential apoptotic marker, revealed that this marker is not significantly affected at the 2- or 4-h time points compared with controls. With increased withdrawal time (6 or 18 h) plasma and brain ketamine levels are approximately zero, however, neuronal cell death is significantly increased. Figure 1B shows that enhanced apoptosis (about a 48% increase) was apparent in ketamine-treated rat pups that were sacrificed 6 or 18 h after last ketamine administration compared with controls.

At the EM level, the direct evidence of increased neuronal cell death after multiple ketamine injections to PND-7 rats ($20 \text{ mg/kg} \times 6$), was confirmed. Figure 2 shows representative nuclear fragmentation (Fig. 2B), and nuclear condensation (Fig. 2C). The increased nuclear fragmentation and condensation represent advanced states of apoptosis, compared with normal cortical neurons in control animals with an intact cytoplasm and nuclear membrane (Fig. 2A)

To study the vulnerability of the immature rat brain to ketamine-induced neurotoxicity, PND-7 rats were exposed to 5, 10, or 20 mg/kg ketamine in single or multiple injection (three or six times), respectively, with 2-h intervals between injections. Several histological approaches that include caspase-3 immunostaining and Fluoro-Jade C staining were applied to evaluate the neurodegeneration. Multiple ketamine injections to PND-7 rats ($20 \text{ mg/kg} \times 6$) produced the most severe neuronal damage as indicated by a large increase in the number of caspase-3-positive neurons in neocortical areas, especially in layers II and III of the frontal cortex (Fig. 3D), when compared with controls (Fig. 3A). However, no significant effects were observed in the animals injected either one or three times with 20 mg/kg ketamine (Fig. 3E). Meanwhile, enhanced apoptotic cell death was not detected in layers II and III in the frontal cortex of rat brains exposed to 5 or 10 mg/kg of ketamine in single or multiple injections (three or six times) compared with controls (Fig. 3). As was the case for the caspase-3 expression pattern, significantly increased numbers of Fluoro-Jade C-positive neuronal cells (Fig. 4) were observed in the frontal cortex of animals exposed to multiple ketamine injections ($20 \text{ mg/kg} \times 6$), but not in animals injected either 1 or 3 times with 20 mg/kg ketamine or

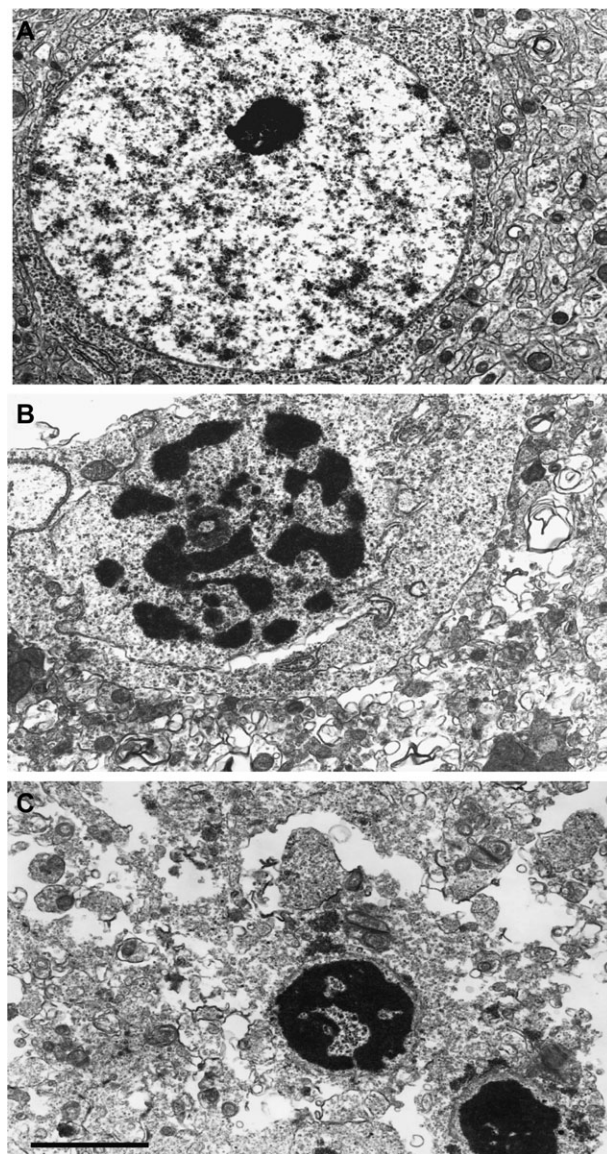


FIG. 2. Electron micrographs (EM) show a normal neuron with intact cytoplasm and nuclear membrane from a PND-7 control rat (A). EMs also show typical nuclear fragmentation (B) and nuclear condensation (C) that represent advanced states of apoptosis in layers II and III of the frontal cortex from ketamine- ($20 \text{ mg/kg} \times 6$ injections) treated rat brain. Scale bar = $0.64 \mu\text{m}$

animals exposed to 5 or 10 mg/kg ketamine in a single or multiple injections.

In the present study, several major brain regions including striatum, hippocampus, thalamus, and amygdala were examined for indicators of neurotoxicity induced by ketamine ($20 \text{ mg/kg} \times 6$ injections). Only a few caspase-3-positive neuronal cells were observed in saline-treated control animals in these brain regions, however, increased numbers of caspase-3-positive apoptotic neural cells were apparent in ketamine-treated rat brains. Statistical analyses of these data (Table 1) indicated that ketamine administration ($20 \text{ mg/kg} \times 6$ injections)

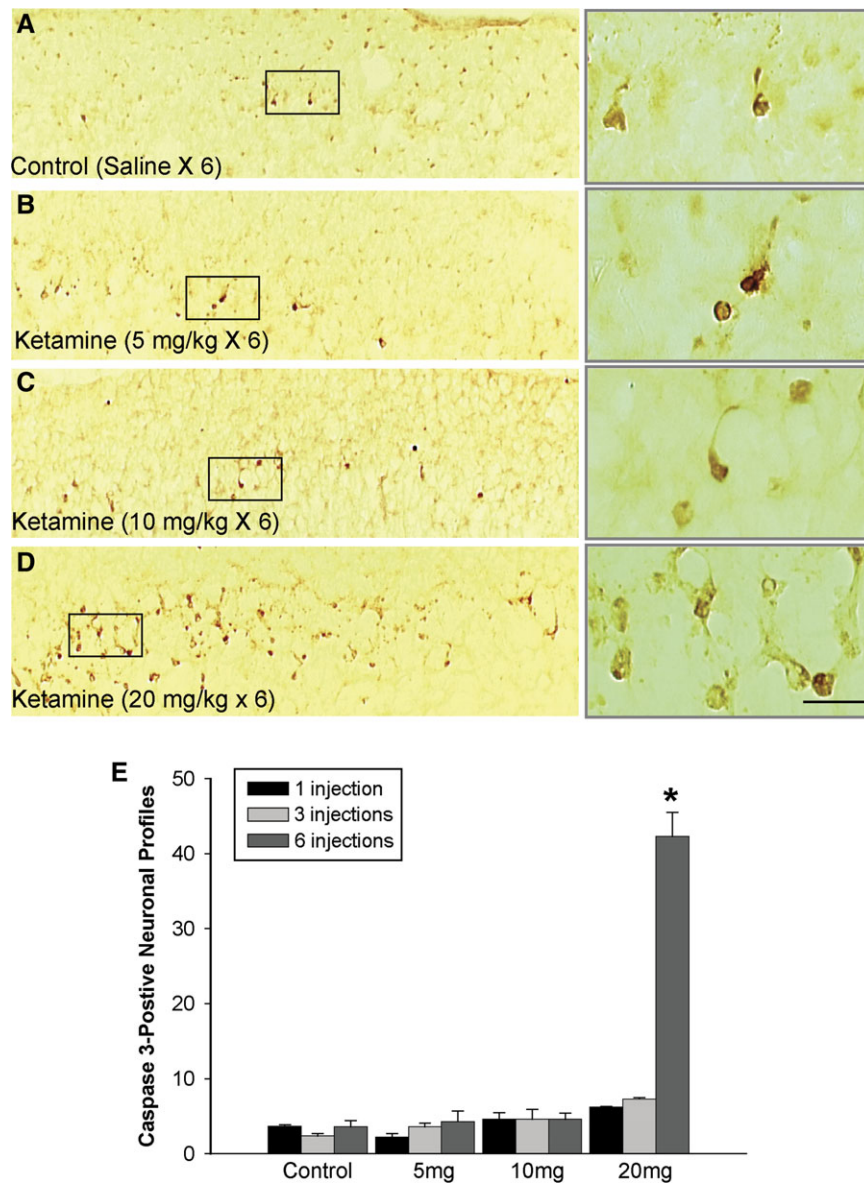


FIG. 3. Representative immunocytochemical pictures and quantitative analysis of caspase-3 in the frontal cortex from control and ketamine-treated PND-7 rats. Only a few caspase-3-positive neuronal profiles were observed in layers II and III of the frontal cortex in PND-7 rats exposed to 5 and 10 mg/kg of ketamine in a single or multiple (three or six) injections ($n = 5$ per group). Significant enhancement of neurotoxicity was detected from animals treated with six injections of 20 mg/kg ketamine, but not in animals treated with one or three injections of 20 mg/kg ketamine. For each condition, at least five animals were randomly assigned. Data are presented as means \pm SEM. A probability of $*p < 0.05$ was considered significant (one-way ANOVA). Scale bar = 50 μ m.

resulted in a 3-fold increase in striatum, 2.5-fold increase in hippocampus, 2.3-fold increase in thalamus, and 3-fold increase in amygdala of caspase-3-positive neural cells when compared with controls. It should be noted that the most extensive apoptotic cell death as indicated by caspase-3 immunostaining was observed in the frontal cortex (about 10-fold), therefore, this extremely vulnerable brain region was utilized for an *in situ* hybridization study to detect potential alterations in NMDA receptor NR1 subunit messenger signaling after ketamine exposure (20 mg/kg \times 6 injections).

Ketamine-Induced Neurodegeneration and Altered NMDA Receptor Expression

Because ketamine is an NMDA receptor antagonist, it seemed possible that the localization of the most severe brain damage (apoptotic neurons) in the frontal cortex might correspond to alterations in NMDA receptor expression. In both controls and ketamine-treated (20 mg/kg \times 6 injections) rats, NMDA receptor NR1 subunit mRNA was prominent (Figs. 5A and 5B). The autoradiograph grain density (labeling) for NR1 subunit mRNA was upregulated in rats that were

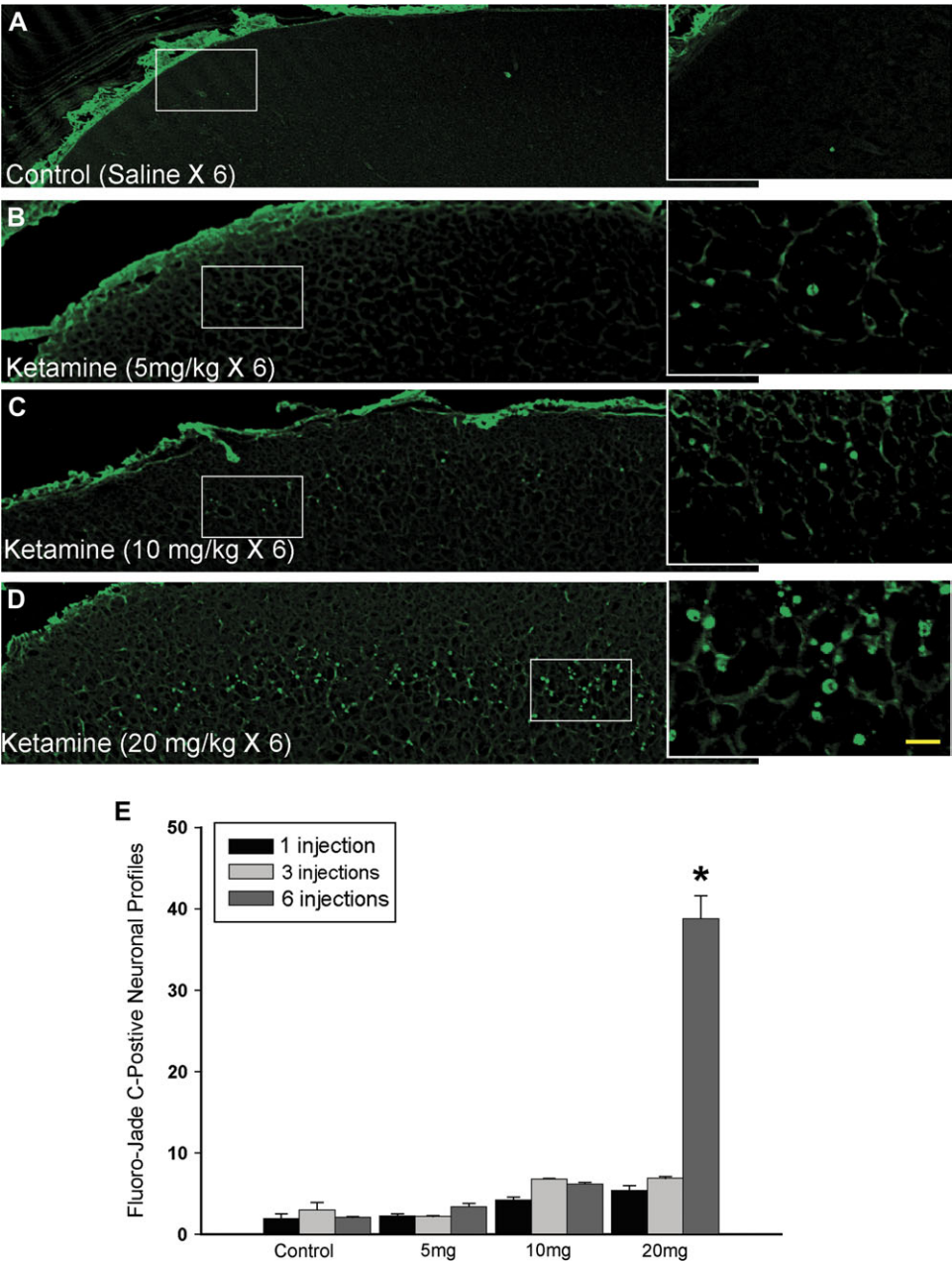


FIG. 4. Neurotoxicity of ketamine as indicated by Fluoro-Jade C staining in layers II and III of the frontal cortex. No significant neurotoxic effects were observed when the animals were exposed to ketamine (5 or 10 mg/kg \times 1, 3, or 6 injections) compared with controls. However, an increased number of Fluoro Jade C-positive neuronal profiles were apparent when the animals were exposed to six injections of 20 mg/kg ketamine, but not in animals treated with one or three injections of 20 mg/kg ketamine. For each condition, at least five animals ($n = 5$ per group) were randomly assigned. Data are presented as means \pm SEM. A probability of $*p < 0.05$ was considered significant (one-way ANOVA). Scale bar = 50 μ m.

treated with multiple ketamine injections (Fig. 5B). The emulsion autoradiograph density of NR1 mRNA in layers II and III appears to correspond to the frontal cortex area from ketamine-treated rats that was most heavily damaged as revealed by caspase-3 immunostaining and Fluoro-Jade C staining. Quantitative analysis of the NR1 *in situ* hybridization signal indicates that a significant effect was observed between controls and ketamine-treated rats (Fig. 5C).

DISCUSSION

Ketamine, a noncompetitive NMDA receptor antagonist, is a widely used dissociative anesthetic agent, and blockade of NMDA receptors is known to cause neurotoxicity in some instances (Ikonomidou *et al.*, 1999; Wang *et al.*, 2005, 2006). In order to better determine if the neurotoxicity associated with ketamine in the developing rat has clinical relevance, and to

TABLE 1
**The Number of Caspase-3-Positive Profiles (mean \pm SEM/
 section) in Several Major Brain Regions**

	Frontal cortex	Striatum	Hippocampus	Thalamus	Amygdala
Control	3.6 \pm 0.8	4.4 \pm 1.2	6.5 \pm 2.8	4.2 \pm 0.7	2.2 \pm 1.1
Ketamine	42.3 \pm 3.2*	13.6 \pm 3.2*	16.2 \pm 4.1*	9.9 \pm 1.0*	7.4 \pm 0.8*

Note. * $p < 0.05$ versus control, $n = 5$ per group.

dissect underlying mechanisms and intracellular pathways that mediate cell death responses, the effects of ketamine need to be systematically examined in other species under appropriate *in vivo* conditions during development.

Ketamine is distributed rapidly after intravenous administration with a bioavailability of 93% (Grant *et al.*, 1981). Ketamine undergoes N-demethylation to its primary active metabolite, norketamine. Norketamine is 1/3 to 1/5 as potent as ketamine but may provide prolonged analgesic action (Kohrs and Durieux, 1998). In the present study, ketamine levels in the plasma and brain tissue were highest at the initial sampling point, 5 min, after which concentrations decreased rapidly, dropping to undetectable levels at approximately 6 h after the last ketamine injection with a calculated elimination half-life of 0.7–0.9 h. The initial concentration of ketamine in brain was lower than that in plasma, but was greater thereafter because of the slower elimination from this tissue. Meanwhile, the metabolite norketamine in plasma and brain tissue peaked approximately 2 h after the last ketamine injection and decreased to undetectable levels approximately 6 h after the last ketamine injection. Norketamine levels were consistently lower in brain tissue than in plasma. The half-life of norketamine was approximately 3–6 h in plasma and brain tissue from ketamine-treated rat pups. Taken together, these results suggest that plasma and brain ketamine clearance is more rapid in immature rats than indicated in clinical reports, but more prolonged exposure to norketamine occurs (Clements and Nimmo, 1981; Grant *et al.*, 1981).

Nucleosomal DNA fragmentation is a characteristic of apoptotic nuclei. The potential neuroapoptotic effect of ketamine was assessed by measuring DNA associated with nucleosomal histones using a specific two-site ELISA. Although the concentrations of ketamine in plasma and brain were the highest or maintained at certain levels from 5 min to 4 h after ketamine administration, no significant neuroapoptotic effects were detected 2 and 4 h after the last ketamine injection. In contrast, beginning approximately 6 h through 18 h after the last injection when ketamine levels in plasma and brain had decreased to nearly zero, enhanced apoptosis was apparent (approximately 2-fold increase). These data suggest that enhanced apoptotic cell death is not directly associated with the *in situ* blood and brain ketamine levels, but may reflect some indirect or compensatory mechanisms, such as altered NMDA receptor subunit mRNA expression.

In the present study, EM observations showed typical nuclear condensation and fragmentation in layers II and III of the frontal cortex of the ketamine-treated rat brain that represent advanced states of apoptosis. Although it was not the intent of this study to absolutely distinguish between apoptosis and necrosis, the EM data coupled with the results from the Cell Death Detection ELISA and the increased number of caspase-3-positive neurons after multiple ketamine administrations, support the hypothesis that ketamine-induced neuronal cell death in the developing rat brain is mainly apoptotic in nature.

Anesthetic drugs also produce dose-dependent cellular effects (Anand *et al.*, 2004; Wang *et al.*, 2005, 2006). Ketamine may act as an anti-inflammatory agent at subanesthetic concentrations (Roytblat *et al.*, 1998; Zilberstein *et al.*, 2002), whereas higher concentrations produce a nonspecific cytostatic effect (Lewis *et al.*, 2001). High doses of ketamine also promote seizures (Lees, 1995), a property shared by other anesthetic agents (Roytblat *et al.*, 1986). In the present study, no significant increase in apoptotic neurodegeneration in the frontal cortex was detected in animals exposed to a single or multiple injections (three or six) of ketamine at 5 or 10 mg/kg. However, a remarkable increase in apoptotic neurodegeneration was observed in layers II and III of the frontal cortex and several major brain regions including the striatum, hippocampus, thalamus, and amygdala in animals treated with six injections of 20 mg/kg ketamine, as revealed by caspase-3 immunostaining. Multiple ketamine injections (20 mg/kg \times 6) produce the most severe neuronal damage (~10-fold increase) in the frontal cortex versus a 3-fold increase in the striatum, a 2.5-fold increase in the hippocampus, a 2.3-fold increase in the thalamus, and a 3-fold increase in the amygdala. These data are consistent with previous reports that exposure of the developing brain to NMDA antagonists such as ketamine or PCP results in widespread and dose-dependent apoptotic neurodegeneration (Ikonomidou *et al.*, 1999, 2001; Jevtovic-Todorovic *et al.*, 2003; Olney *et al.*, 2002). These data also suggest that the frontal cortex is the brain region most vulnerable to ketamine-induced neurotoxicity.

Neurodegenerative changes in the developing brain occur following prolonged exposure to anesthesia in neonatal rats (Ikonomidou *et al.*, 1999; Olney *et al.*, 2002), confirming earlier findings from exposure to halothane or NMDA receptor blockade (Griesbach and Amsel, 1998; Uemura *et al.*, 1985). Repeated ketamine injections increase neuronal cell death in multiple areas of the neonatal rat brain (Hayashi *et al.*, 2002). In the present study, a significantly increased number of caspase-3- and Fluoro-Jade C-positive neurons were observed in the frontal cortex in animals exposed to six injections of 20 mg/kg ketamine, but not in animals administered one or three injections of 20 mg/kg ketamine. These findings were confirmed by previous *in vivo* and *in vitro* studies in the developing nonhuman primate (Slikker *et al.*, 2007; Wang *et al.*, 2006). In the monkey *in vivo* study, PND-5 monkeys were evaluated after 3 or 24 h of ketamine anesthesia. The 24-h

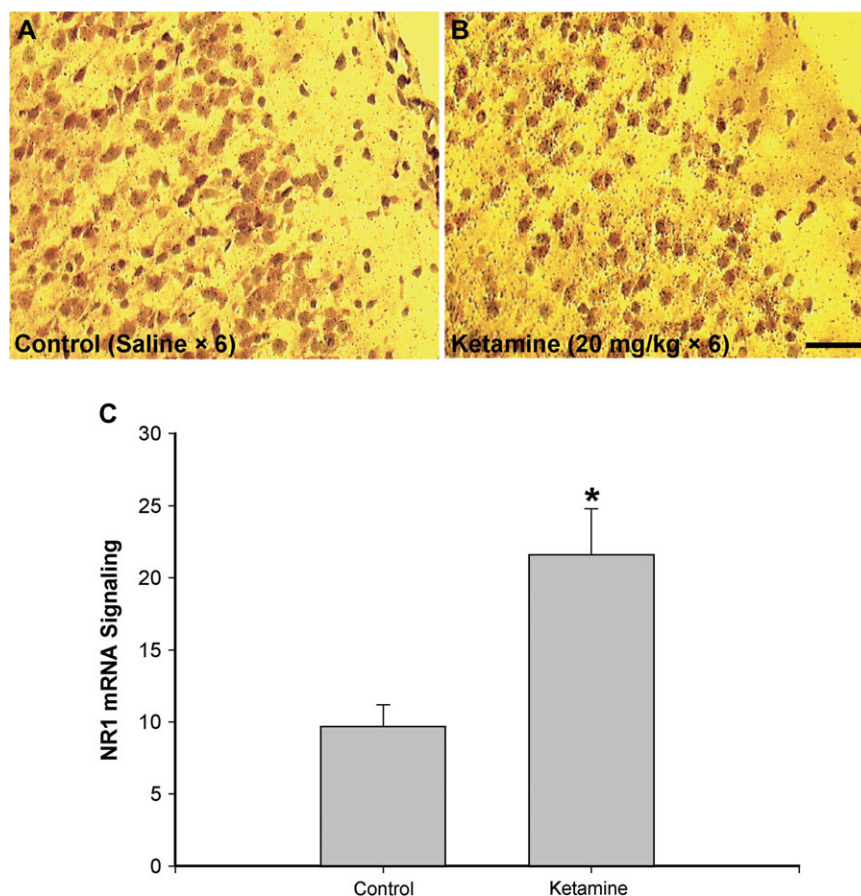


FIG. 5. NMDA receptor NR1 subunit mRNA abundance in the frontal cortex of PND-7 rats. The autoradiograph grain density (labeling) for NR1 subunit mRNA was upregulated in ketamine- (20 mg/kg \times 6 injections) treated rat brain (B) compared with control (A). C shows quantitative analysis (relative labeling density) of the effects of ketamine on the *in situ* hybridization signal of NMDAR1 subunit mRNA expression in layers II and III of the frontal cortex. Scale bar = 60 μ m.

duration was selected as a relatively long duration, whereas the 3-h duration more closely approximates typical general pediatric anesthetic episodes. The results indicated that no significant neurotoxic effects were observed if the anesthesia duration was 3 h; however, 24-h anesthetic sessions produced a significant increase in neuronal cell death in the frontal cortex. These data are also consistent with those observed in the time-course studies using a primary cortical culture system established from developing rats and monkeys (Wang *et al.*, 2005, 2006). Therefore, there is no doubt that prolonged exposure of perinatal rat pups to anesthetic and anticonvulsant drugs leads to accelerated neurodegeneration. We propose that potential anesthetic-induced neurotoxicity may depend on the concentrations (doses) of drugs, the duration of the exposure, the route of administration, the receptor subtype activated, the animal species, and the stage of development or maturity at the time of exposure. These findings are important because these concentrations/exposure durations reflect thresholds of exposure for producing neurotoxic effects in the developing nervous system.

The excessive suppression of neuronal activity during synaptogenesis might automatically activate an internal signal for a developing neuron to commit suicide. Accentuated neurodegenerative mechanisms in the immature brain increase neuronal susceptibility to various metabolic events or exposure to anesthetic agents (Bhutta and Anand, 2002). Activation (endogenous glutamate) of upregulated NMDA receptors (after ketamine washout) could result in a toxic accumulation of intracellular free calcium. Associated with Ca^{2+} influx is an increase in ROS that appears to originate in the mitochondria (Johnson *et al.*, 1998; Slikker *et al.*, 2005). The buffering capacity of the mitochondria is exceeded by the increased Ca^{2+} , thus reducing membrane potential and disrupting electron transport, which results in the increased production of reactive free radical superoxide anions [O_2^-] (Slikker *et al.*, 2005, 2007; Wang *et al.*, 2000).

In the present study, by using *in situ* hybridization techniques to detect the relative densities of NMDA receptor NR1 subunits, a potential parallel relationship between enhanced apoptosis and NMDA receptor expression levels

was examined. The data indicated that ketamine exposure resulted in severe neurodegeneration in the frontal cortex, and this pathological change was closely associated with significant upregulation of NMDA NR1 subunit mRNA. The NMDA receptor NR1 subunit is widely distributed throughout the brain and is the fundamental subunit necessary for NMDA channel function. NMDA receptor density has been shown to increase in cultured cortical neurons after exposure to the NMDA receptor antagonists D-AP5, CGS-19755, and MK-801, but not after exposure to the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid/kainate receptor antagonist CNQX (Williams *et al.*, 1992). We hypothesize that continuous blockade of NMDA receptors by ketamine causes a compensatory upregulation of NMDA receptors and this upregulation makes neurons bearing these receptors more vulnerable, after ketamine withdrawal, to the excitotoxic effects of endogenous glutamate, because this upregulation of NMDA receptors allows for the accumulation of toxic levels of intracellular calcium even under normal physiological conditions. This hypothesis is supported in our previous *in vitro* studies by the observation that coadministration of antisense oligonucleotides that specifically target NMDA receptor NR1 subunit mRNA was able to block the neuronal damage induced by ketamine (Wang *et al.*, 2005, 2006).

Taken together, these data demonstrate that ketamine administration results in a dose-related and exposure-duration dependent increase in neuronal cell death during development. Ketamine-induced cell death appears to be apoptotic in nature in the developing rat brain. A significant upregulation of NMDA receptor NR1 subunit signaling was elicited when PND-7 rats were exposed to multiple doses of ketamine (20 mg/kg \times 6 injections). In addition, enhanced apoptosis does not appear to be directly associated with *in situ* plasma and brain ketamine levels, but is closely associated with altered NMDA receptor subunit mRNA expression.

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