

# Blockade of N-Methyl-D-Aspartate Receptors by Ketamine Produces Loss of Postnatal Day 3 Monkey Frontal Cortical Neurons in Culture

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Ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is used as a general pediatric anesthetic. Recent data suggest that anesthetic drugs may cause neurodegeneration during development. The purpose of this study was to determine the robustness of ketamine-induced developmental neurotoxicity using rhesus monkey frontal cortical cultures and also to determine if dysregulation of NMDA receptor subunits promotes ketamine-induced cell death. Frontal cortical cells collected from the neonatal monkey were incubated for 24 h with 1, 10, or 20  $\mu$ M ketamine alone or with ketamine plus either NR1 antisense oligonucleotides or the nuclear factor  $\kappa$ B translocation inhibitor, SN-50. Ketamine caused a marked reduction in the neuronal marker polysialic acid neural cell adhesion molecule and mitochondrial metabolism, as well as an increase in DNA fragmentation and release of lactate dehydrogenase. Ketamine-induced effects were blocked by NR1 antisenses and SN-50. These data suggest that NR1 antisenses and SN-50 offer neuroprotection from the enhanced degeneration induced by ketamine *in vitro*.

**Key Words:** NMDA receptor; ketamine; antisense oligonucleotide; neurodegeneration; *in vitro*; neonatal rhesus monkey.

Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor ion channel blocker, is used as a general pediatric anesthetic. Ketamine is a nonbarbiturate, dissociative anesthetic used during short diagnostic and surgical procedures in infants and toddlers. Recent data from developing rats (Ikonomidou *et al.*, 1999; Jevtovic-Todorovic *et al.*, 2003;

Scallet *et al.*, 2004; Wang *et al.*, 2005b) suggest that anesthetic drugs may cause dose-dependent neurodegeneration. In order to better determine if the neurotoxicity of ketamine in the developing rat has clinical relevance, ketamine should be examined in a nonhuman primate model that more closely mimics the developing pediatric population.

NMDA receptors are involved in a variety of physiological and pathological processes, including memory and learning (Collingridge *et al.*, 1983), neuronal development (D'Souza *et al.*, 1993), epileptiform seizures, synaptic plasticity (Meldrum and Garthwaite, 1990), and acute neuropathologies, such as stroke and other trauma-related events (Beal, 1992). There is also evidence for its involvement in chronic neuropathologies such as Alzheimer's (Cotman *et al.*, 1989), Parkinson's and Huntington's diseases (Greenamyre, 1993; Meldrum and Garthwaite, 1990), and mental illnesses such as schizophrenia and anxiety disorder (Meldrum and Garthwaite, 1990).

Modifications of synaptic efficacy are believed to play an important role in information processing and storage by neuronal networks. The  $\alpha$ -2,8-linked sialic acid polymer (polysialic acid [PSA]) on neural cell adhesion molecules (NCAM) is an important regulator of cell-surface interactions (Muller *et al.*, 1996). PSA-NCAM is also a neuron-specific marker, known to be an NMDA-regulated molecule important in synaptogenesis during development (Wang *et al.*, 2005).

Blockade of NMDA receptors is known to be neurotoxic, but the underlying mechanism is uncertain. Administration of NMDA receptor antagonists, such as ketamine, phencyclidine (PCP), and MK-801, to perinatal rats during a critical time of development has been demonstrated to result in neurotoxicity or neurodegeneration in several major brain areas (Ikonomidou *et al.*, 1999; Scallet *et al.*, 2004). In 1999, Olney *et al.* demonstrated severe widespread apoptotic degeneration throughout the rapidly developing brain of the 7-day-old rat pup after ketamine administration (Ikonomidou *et al.*, 1999).

The main purposes of this study were to determine the robustness of ketamine-induced neurotoxicity using nonhuman

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primate (rhesus monkey) frontal cortical cultures by examining neuropathological and neurobiological outcomes and also to determine if dysregulation of NMDA receptor subunits promotes ketamine-induced cell death. It is proposed that the administration of ketamine during critical developmental periods will result in a dose-related increase in neurotoxicity and loss of neurons (decreased expression of PSA-NCAM) by a mechanism in which a compensatory upregulation of NMDA receptor subunits may be involved. If NMDA receptor NR1 subunit antisense oligonucleotides (ODNs) can protect neurons from ketamine-induced cell death, NMDA receptor dysfunction might be a key mechanism underlying the enhanced neurodegeneration induced by ketamine during development.

## MATERIALS AND METHODS

**Drugs and other materials.** Ketamine hydrochloride (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) was diluted in Dulbecco's Modified Eagle's medium (DMEM). Ketamine was identified and its purity confirmed with high-performance liquid chromatography and mass spectrometry. Antisense and sense (for the NMDA receptor NR1 subunit) ODNs were synthesized by Sigma Genosys Biotechnologies, Inc. (The Woodlands, TX). The media (DMEM) and fetal bovine serum were purchased from Invitrogen (Grand Island, NY). 2-D-L-Aminophosphonovaleric acid (d-APV; a competitive antagonist of the NMDA receptor) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; an AMPA [DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid] and kainate receptor antagonist) were purchased from Tocris Neuramin (Bristol, U.K.). The antagonist of the L-type voltage-sensitive  $\text{Ca}^{2+}$  channel, nifedipine, and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

**Primary cell culture.** All animal procedures were approved by the Institutional Animal Care and Use Committee of the National Center for Toxicological Research and conducted in full accord with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Animal procedures were designed to minimize the number of animals required. Primary frontal cortical cultures were prepared (Wang *et al.*, 2000, 2005) from three (two female and one male) postnatal day (PND) 3 rhesus monkeys (*Macaca mulatta*) on three different days. The experiments were repeated three times ( $n = 3$ ). Briefly, monkeys were deeply anesthetized with ketamine (20 mg/kg, im), dipped in 70% ethanol for sterilization, and sacrificed by exsanguination. The brains were quickly removed and frontal cortex dissected out and placed in cold Hank's solution. Frontal cortical slices were placed in 10-cm culture dishes containing defined serum-free DMEM, cut into small pieces, and then transferred to 15-ml centrifuge tubes. After a brief centrifugation (1000 rpm for 2 min) to pellet the tissue, the supernatant was removed and replaced with medium containing papain (2 mg/ml in DMEM medium) and incubated at 37°C in a shaking water bath for 30 min. After another brief centrifugation (1000 rpm for 2 min), the medium containing papain was aspirated and replaced with 1.5 ml cold Hank's solution without  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . The cells were then dissociated by mechanical agitation through a fire-polished Pasteur pipette. After washout of the papain by centrifugation (1000 rpm for 10 min), cells were distributed and grown on polylysine-coated coverslips in DMEM supplemented with 10% (vol/vol) fetal bovine serum. Glial proliferation was stopped with a mitotic inhibitor, cytosine  $\beta$ -D-arabinofuranoside (beginning on day 3 of culture). After 1 week, these cultures were approximately 50% neuronal.

Cultures were exposed for 24 h to ketamine in the presence and absence of antisense ODN specifically targeted to the NMDA receptor NR1 subunit on day 5 in culture. After the addition of serum and glutamate-containing media (with and without protective agents) or defined serum-free media, neurotoxicity was evaluated after 24 h using various assays as described below. In time course

studies, the cultures were exposed to ketamine for 2, 6, 12, or 24 h, and the neurotoxicity was examined 24 h after ketamine washout. In withdrawal (ketamine washout) time course studies, the cultures were exposed to ketamine for 24 h, and the neurotoxicity was examined 2, 6, 12, and 24 h after ketamine washout.

**Immunocytochemistry and nuclear staining.** A mouse monoclonal antibody to PSA-NCAM (1:500) was used to identify neurons, and a rabbit polyclonal antibody to glial fibrillary acidic protein (GFAP; 1:200) was used to identify glial cells. Cultures were rinsed with phosphate-buffered saline (PBS), fixed with ice-cold (4°C) 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, and an indirect immunofluorescence technique was used to visualize immunoreactivities. The cells were incubated with primary antibodies (diluted in PBS/0.5% bovine serum albumin [BSA] solution) at 4°C overnight. Bound antibodies were revealed with fluorescein isothiocyanate-conjugated sheep anti-mouse IgG second antibody (diluted 1:80 in PBS/0.5% BSA solution) or rhodamine-conjugated sheep anti-rabbit IgG second antibody (diluted 1:40 in PBS/0.5% BSA solution). The cells were examined with an Olympus light microscope equipped with epifluorescence.

Cultured cells were counterstained with bisbenzimidazole solution (Hoechst 33258; Sigma). Bisbenzimidazole (0.1  $\mu\text{g}/\text{ml}$ ) was dissolved in PBS/glycerol (1:1). After applying two drops of bisbenzimidazole solution to the microscope slide, the coverslips with cells were mounted onto the slides and observed under a fluorescence microscope at an excitation wavelength of 365 nm.

**Fragmented DNA detection by enzyme-linked immunosorbent assay.** Nucleosomal DNA fragmentation is characteristic of apoptotic nuclei. The presence of fragmented DNA was assessed by measuring the DNA associated with nucleosomal histones using a specific two-site enzyme-linked immunosorbent assay (ELISA) with an anti-histone primary antibody and a secondary anti-DNA antibody according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). Briefly, the cells were homogenized in 3 ml lysis buffer (provided by the 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  $\mu\text{l}$  from each sample was transferred to a 96-well plate precoated with anti-histone antibody to which 80  $\mu\text{l}$  immunoreagent mix was added. After incubation and washes, the wells were treated with the chromogen 2,2'-azinobis(3-ethylbenzothiazoline) sulfonic acid as a substrate. The intensity of the color that developed was measured at 405 nm, while that at 490 nm was used as a blank (reference wavelength).

**MTT reduction cell viability assay.** The MTT dye is metabolized by viable mitochondria to a colored product and can then be detected photometrically. Thus, the extent of MTT metabolism is an indicator of mitochondrial function. Briefly, after the removal of media for use in the lactate dehydrogenase (LDH) assay (see below), 100  $\mu\text{l}$  MTT (5 mg/10 ml of medium) was added to each well, and the plate was incubated for 4 h at 37°C. The MTT solution was removed and dimethyl sulfoxide (100  $\mu\text{l}$ ) was added to each well; the color intensity was assessed with a plate reader at 590 nm.

**LDH release.** LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but released from necrotic cells with damaged membranes. After exposure (24 h) to serum and glutamate-containing media (cultures previously treated with different concentrations of ketamine or control media), the media was collected and assayed for LDH activity using a cytotoxicity detection kit from Roche Applied Science (Indianapolis, IN). Briefly, LDH catalyzes the conversion of lactate to pyruvate upon reduction of  $\text{NAD}^+$  to  $\text{NADH}/\text{H}^+$ ; the added tetrazolium salt (yellow) is then reduced to formazan (red). The amount of formazan formed correlates with LDH activity. The formazan product was measured with a microtiter plate reader at 490 nm.

**TUNEL assay.** This assay is used to identify apoptotic cells *in situ*. It relies on the detection of fragmented DNA strands, but because fragmentation can occur via nonapoptotic mechanisms, it is not absolutely specific for apoptosis. Following exposure (24 h) to serum and glutamate-containing media (cultures

previously treated with different concentrations of ketamine or control media), the cells were rinsed with PBS, fixed with ice-cold (4°C) 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.2, and processed for evaluation of nuclei containing fragmented DNA *in situ*. Terminal deoxynucleotidyl transferase, a template-independent polymerase, was used to incorporate biotinylated nucleotides at the sites of DNA breaks as previously described (Johnson *et al.*, 1998). The terminal deoxynucleotidyl transferase dUTP-biotin nick-end-labeling (TUNEL)-positive cells were then photographed with an Olympus light microscope.

**Western blot analysis.** Western blot analysis was used for the quantification of protein expression levels. The cells were placed in lysis buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS] in normal PBS; pH 6.8) containing a protease inhibitor cocktail (10 µl/ml; Sigma-Aldrich). The homogenate was centrifuged at 14,000 × g for 10 min, and the supernatant was collected and stored at -80°C until assayed. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (10 µg) from the supernatant fraction were loaded on each lane and run on SDS-polyacrylamide gels with a Tris-glycine running buffer system and then transferred to a polyvinylidene difluoride membrane (0.2 µm) in a Mini Electrottransfer Unit (Bio-Rad, Hercules, CA). The blots were probed with anti-PSA-NCAM (1:2000, monoclonal), anti-NR1 (1:300, monoclonal, BD Biosciences Pharmingen, San Diego, CA), and anti-actin (1:3000, monoclonal, Chemicon, Temecula, CA). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG using enhanced chemiluminescence Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ). The bands corresponding to PSA-NCAM, NR1, and β-actin were scanned and densitometrically analyzed using an automatic image analysis system (Alpha Innotech Corporation, San Leandro, CA). These quantitative analyses were normalized to β-actin (after stripping) and expressed as means ± SEMs. One-way ANOVA was used to compare the levels of each protein among the different treatment groups.

**Electrophoretic mobility shift assay.** Cultures were exposed to ketamine for 24 h, and then ketamine was washed out and replaced with serum-containing media for another 24 h. SN-50 (2.5µM) and SN-50-Control (2.5µM; [SN-50-Control has a chemical and molecular structure similar to SN-50, but its functional residues are replaced]) were applied. The medium was then removed, and the attached cells were washed with PBS; nuclear extracts were prepared according to published methods (Dignam *et al.*, 1983; Osborn *et al.*, 1989) with some modifications. Briefly, cells were homogenized in buffer A (10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES], 10mM KCl, 0.1mM ethylenediaminetetraacetic acid [EDTA], 0.1mM ethyleneglycotetraacetic acid [EGTA], 1mM phenylmethylsulfonyl fluoride, 2 µg/ml antipain, 2 µg/ml chymostatin, 2 µg/ml pepstatin, and 2 µg/ml leupeptin [pH 7.8]) with approximately 15 strokes of a 1-ml-manual Wheaton Tenbrock tissue grinder (Fisher Scientific, Houston, TX). The lysate was microcentrifuged (8000 rpm for 2 min) to collect nuclei. Nuclear protein was extracted by suspending the nuclei in extraction buffer B (20mM HEPES, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride, 2 µg/ml antipain, 2 µg/ml chymostatin, 2 µg/ml pepstatin, and 2 µg/ml leupeptin [pH 7.9]) for 20 min (4°C). The nuclei were subjected to centrifugation, and the supernatant was divided into aliquots.

Double-stranded DNA containing the sequence corresponding to the classical nuclear factor κB (NF-κB) consensus site (5'-AGTTGAGGG-GACTTTCAGGC-3', Santa Cruz Biotechnology, Inc. CA) was end labeled with (γ-<sup>32</sup>P)ATP using T4 kinase (Invitrogen, Rockville, MD). Unincorporated nucleotides were removed using two Sephadex G-50 columns (Amersham Biosciences). Binding reactions were carried out in Tris-HCl, 60mM KCl, 1mM EDTA, 1mM dithiothreitol, 10% glycerol, 2 µg poly(dIdC), 15 µg nuclear extract, and 0.5 ng <sup>32</sup>P-labeled ODN probe (50,000 cpm) at room temperature for 20 min. Gels were dried and subjected to autoradiography, and the radiographs were analyzed by an automatic image analysis system (Alpha Innotech Corporation).

**Statistical analysis.** In these studies, the experimental units were the animals from which the tissues were collected, therefore, *n* = 3. All data were

analyzed using a parametric one-way ANOVA with the Holm-Sidak method for individual differences. The null hypothesis was rejected at a probability level of *p* < 0.05.

**Application of antisense ODNs.** To test whether the administration of antisense ODNs targeted to specific NMDA receptor subunits could block the upregulation of the NMDA receptor subunit mRNAs and proteins, antisense ODNs targeted to NR1 subunit were used in forebrain cultures. The experimental protocol was as follows: the control cultures (group 1) were maintained in normal culture medium for 7 days. For groups 2–4, cultures were maintained in normal culture conditions for 5 days; group 2 cultures were treated with ketamine (10µM) for 24 h, then ketamine was washed out and replaced with serum-containing media for another 24 h. Groups 3 and 4 were treated with ketamine (10µM) plus 2µM antisense ODN for NR1 (group 3) or sense ODN for NR1 (group 4) for 24 h. Ketamine and the ODNs were then washed out and replaced with serum-containing media for another 24 h. All the cells were harvested on day 7. The ODNs used to block the synthesis of the NR1 were as follows: an 18-mer antisense oligodeoxynucleotide corresponding to nucleotide 4–21 (5'-CAGCAGGTGCATGGT-GCT) of the NR1 subunit mRNA (which directly follows the translation initiation codon) and sense (5'-AGCACCATG-CACCTGCTG) ODNs for NR1. The sequence of the antisense ODN was chosen to target the 5' coding region of the NMDAR1 subunit mRNA, and this sequence has proven effective and specific in previous studies (Lai *et al.*, 2000; Roberts *et al.*, 1998; Wahlestedt *et al.*, 1993; Wang *et al.*, 2005a,b).

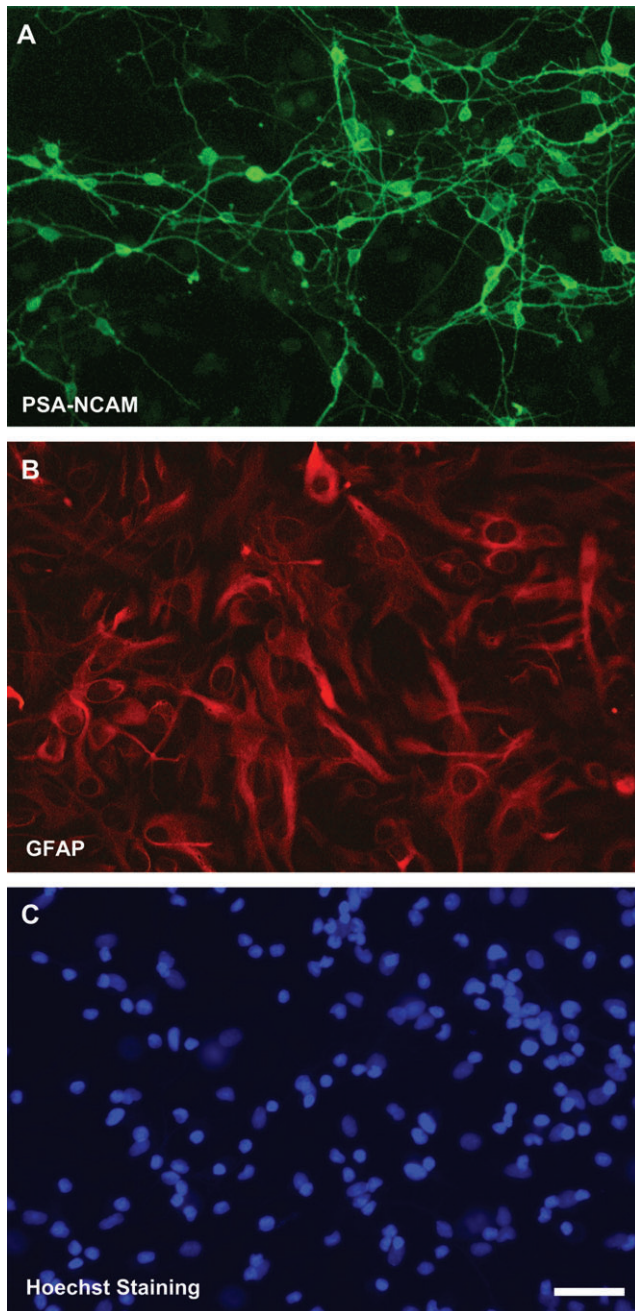
## RESULTS

The use of immunofluorescent staining of PSA-NCAM, GFAP (a glial marker), and nuclear staining by Hoechst 33258 in control monkey frontal cortical cultures revealed that more than 50% of the cells were neurons. Figure 1A illustrates the neuron-specific staining of cultured cells with PSA-NCAM. Figure 1B shows the GFAP-labeled astrocytes, and Figure 1C reveals the total number of the cells (nuclei) in the field, as visualized with a fluorescence microscope.

### *Assessment of Ketamine-Induced Neurotoxicity: Dose Response and Time Course*

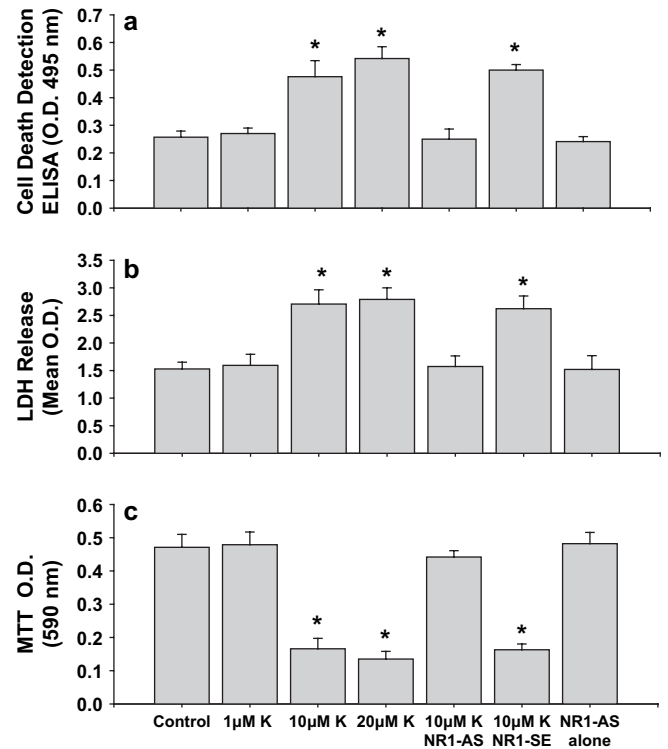
This study sought to define the neurotoxicological effects of treatment with ketamine, a noncompetitive NMDA receptor antagonist, and to facilitate the study of potential mechanisms underlying this toxicity. In these experiments ketamine produced concentration-dependent (1, 10, and 20µM for 24 h) cell death characterized by DNA fragmentation, LDH release, and the MTT and TUNEL assays in cultured frontal cortical cells from PND-3 rhesus monkeys.

Nucleosomal DNA fragmentation is a characteristic of apoptotic nuclei. Figure 2a shows that ketamine treatment of frontal cortical cultures (after ketamine washout, cultures were maintained in serum and glutamate-containing media for 24 h) results in a dose-related increase in neuronal death as indicated by an ELISA for histone-associated DNA fragmentation. No significant neurotoxic effects were found after the administration of 1µM ketamine. However, enhanced apoptosis (~42%) was apparent in cultures treated with 10 or 20µM ketamine.



**FIG. 1.** Immunofluorescence micrographs of monkey frontal cortical culture. (A) Neuron-specific staining of cultured cells with PSA-NCAM as revealed by immunofluorescence of anti-mouse IgG conjugated to fluorescein isothiocyanate. (B) Glia-specific staining of cultured cells with GFAP as revealed by immunofluorescence of anti-rabbit IgG conjugated to rhodamine. (C) Hoechst 33258 nuclear staining reveals the total number (nuclei) of the cells in the field; scale bar, 50  $\mu$ m.

The release of LDH into the media occurs with the loss of plasma membrane integrity, a process most often associated with necrotic cell death. Administration of ketamine (10 and 20 $\mu$ M) produced a remarkable increase (~40%) in the release

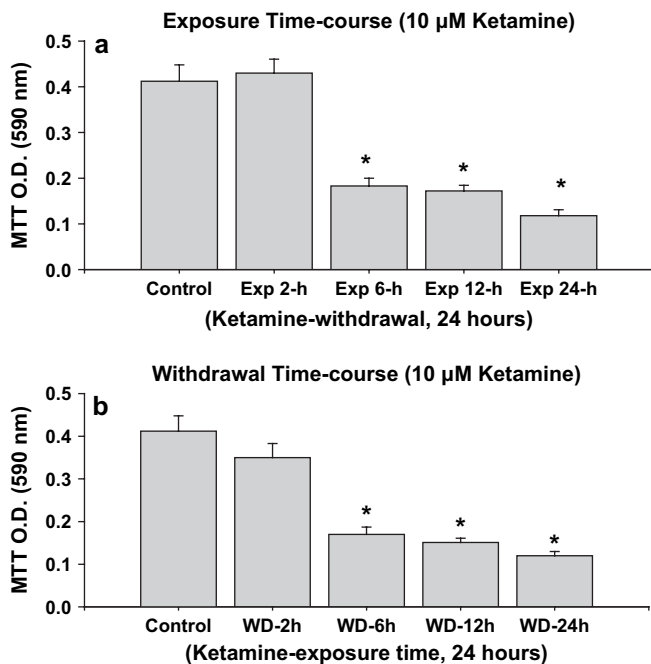


**FIG. 2.** Quantitative analyses of ketamine-induced neurotoxicity and the protective effect of NR1 antisense ODN as assessed by (a) cell death ELISA that measures nucleosomes, (b) LDH release, and (c) MTT assays. Each condition was assessed at least in triplicate, and experiments were repeated three times independently. Data are presented as means  $\pm$  SDs. A probability of  $*p < 0.05$  was considered significant (one-way ANOVA with Holm-Sidak test). K = ketamine; NR1-AS = NR1 antisense ODNs; NR1-SE = NR1 sense ODNs.

of LDH (Fig. 2b). No significant effect was observed in the release of LDH in the 1 $\mu$ M ketamine-treated cultures.

The MTT assay is an important indicator of mitochondrial function and has been used as a marker of cell survival. The MTT assay was applied in this study to monitor the levels of total cell loss (both necrotic and apoptotic). Figure 2c shows that ketamine at 10 and 20 $\mu$ M resulted in a substantial decrease (~75%) in mitochondrial metabolism of MTT into formazan. No significant effect was observed when the ketamine concentration was 1 $\mu$ M.

In the exposure time course study (Fig. 3a), assessment of the effects of ketamine on mitochondrial viability (MTT assay) revealed that this marker is not significantly affected after a 2-h exposure. With increased exposure time, this loss of viability increased to about 58% at 6 h and gradually to about 75% at 24 h. The withdrawal time course (ketamine washout) for ketamine induction of cell death was also evaluated using the MTT assay. Figure 3b shows that there is no significant difference between control and ketamine-treated cells after a washout at 2 h, but that mitochondrial viability was significantly attenuated at 6, 12, and 24 h after ketamine washout.

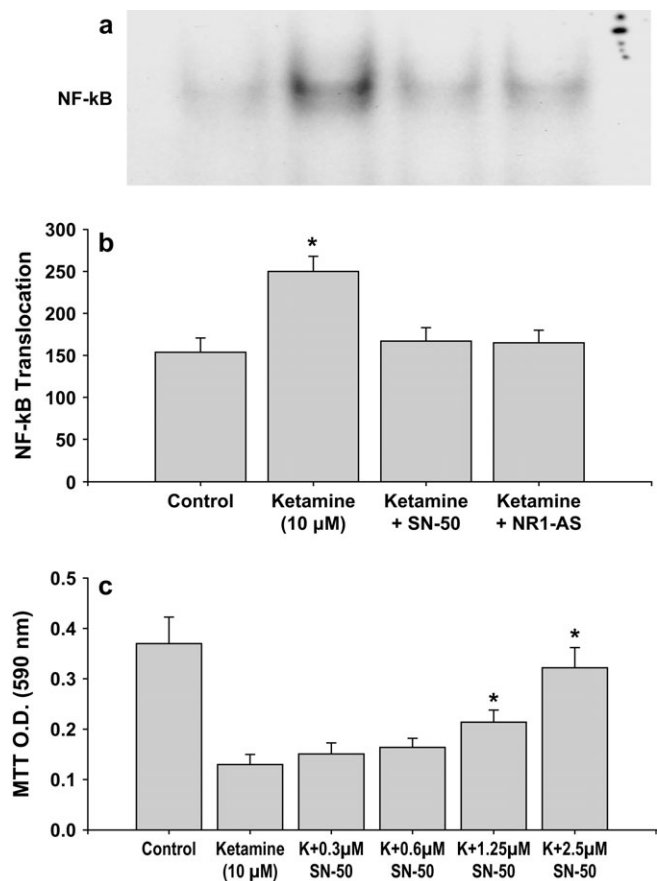


**FIG. 3.** Time course analyses for the effect of ketamine (10 $\mu$ M) on the ability to reduce MTT uptake. In exposure time course (a) the cultures were exposed to ketamine for 2, 6, 12, and 24 h individually, and the neurotoxicity was examined 24 h after ketamine washout using the MTT assay. The withdrawal time course (ketamine washout; b) indicates that there was no significant effect after ketamine washout at 2 h, but at 6, 12, and 24 h, the effects of prior ketamine exposure become evident. Each condition was assessed at least in triplicate, and experiments were repeated three times independently. Data are presented as means  $\pm$  SDs. A probability of  $*p < 0.05$  was considered significant (one-way ANOVA with Holm-Sidak test); WD = withdrawal.

#### The Effect of NF- $\kappa$ B Translocation on Ketamine-Induced Neuronal Cell Death

The possible relationship between ketamine-induced cell death and potential intracellular mediators (NF- $\kappa$ B translocation) was examined using an electrophoretic mobility shift assay. Nuclear protein extracts from control and treated cultures were tested in this assay using a  $^{32}$ P-labeled ODN containing the classical NF- $\kappa$ B consensus sequence. Figure 4a shows a representative gel in which nuclear protein from control and treated cultures retards the migration of the labeled NF- $\kappa$ B binding sequence. Ketamine (10 $\mu$ M) caused an approximately 40% increase in the density of this band relative to the control (Figs. 4a and 4b).

To investigate the role of NF- $\kappa$ B in ketamine-induced cell death, the pharmacological “antagonist” SN-50 a peptide inhibitor of NF- $\kappa$ B transport, was used. SN-50 dose dependently protected against the neurotoxic effect of ketamine as indicated by the MTT assay. These data are summarized in Figure 4c. No significant protective effects were observed by the coadministration of ketamine (10 $\mu$ M) with 0.3 or 0.6 $\mu$ M SN-50. A significant protective effect began at 1.25 $\mu$ M and was maximal (~90% protection) at 2.5 $\mu$ M. Higher concentra-

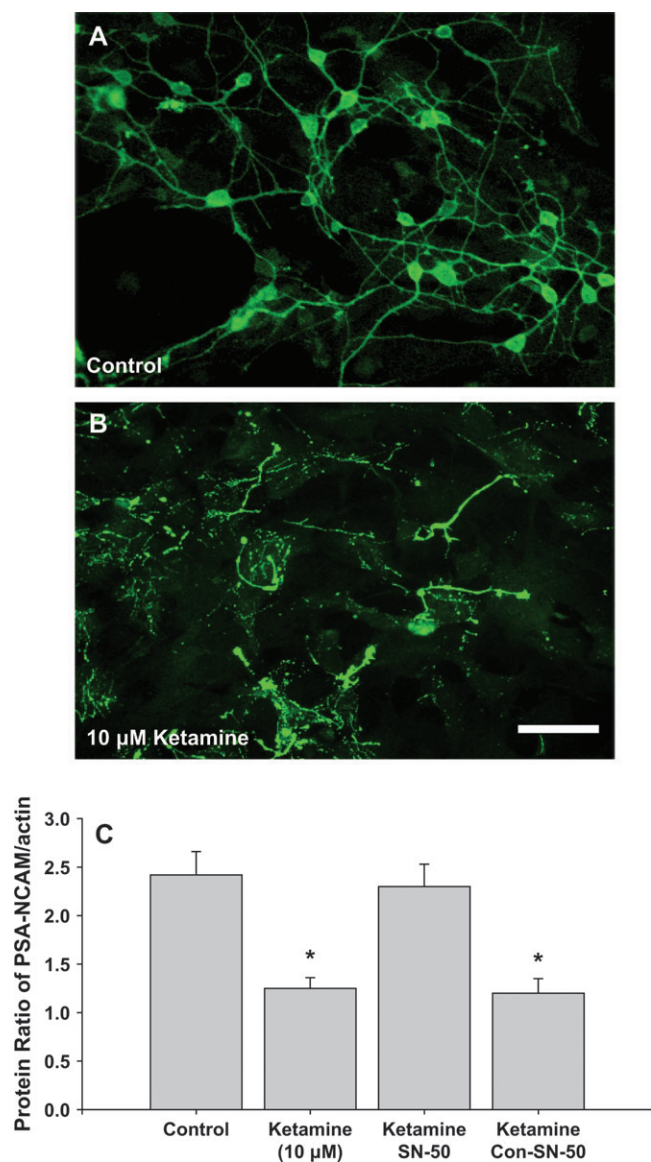


**FIG. 4.** Inhibition of ketamine-induced nuclear translocation of NF- $\kappa$ B by SN-50 and NR1 antisense ODNs. Top panel (a) shows a representative electrophoretic mobility shift assay, where each lane corresponds to the conditions shown in the middle panel (b). The bottom panel (c) indicates that SN-50 protected against the neurotoxic effect of ketamine as indicated by the MTT assay in a dose-dependent manner. No significant protective effects were observed with 0.3 or 0.6 $\mu$ M, but protection began at 1.25 $\mu$ M and was maximal at 2.5 $\mu$ M. Panels (b) and (c) show the data as means  $\pm$  SDs from three identical experiments. A probability of  $*p < 0.05$  was considered significant (one-way ANOVA with the Holm-Sidak test).

tions of SN-50, beginning with 10 $\mu$ M, were actually neurotoxic when administered alone and are therefore not shown.

To better understand the nature of ketamine-induced neurotoxicity, PSA-NCAM (neuronal-specific marker) immunostaining images were acquired from control and ketamine-treated (10 $\mu$ M) cultures. In the control culture, PSA-NCAM immunoreactivity (Fig. 5A) was intense on the surface of cell bodies and processes of neurons. Figure 5B shows that ketamine treatment dramatically diminished PSA-NCAM immunoreactivity. PSA-NCAM expression was weak and characterized by typical residue pieces, fragmentation, and neuronal shrinking.

To determine whether decreased PSA-NCAM expression is associated with ketamine-induced loss of cortical neurons or to local NMDA receptor blockade (as determined by polysialyl transferase activity), Western blot analyses of PSA-NCAM and the protective effects of the peptide blocker of NF- $\kappa$ B



**FIG. 5.** Effect of ketamine and SN-50 on the decrease in PSA-NCAM expression in monkey frontal cortical cultures. PSA-NCAM immunoreactivity was intense in the control culture (A) and diminished in the ketamine-treated culture (B); scale bar, 50  $\mu\text{m}$ . Densitometry measurements were used to calculate a ratio of PSA-NCAM to actin (by stripping the membranes) in each lane in three independent experiments, and the data are shown as the means  $\pm$  SDs of the ratios (C). SN-50 (2.5 $\mu\text{M}$ ) effectively prevented PSA-NCAM reduction induced by ketamine. No protective effect was observed from the inactive control peptide for SN-50 (2.5 $\mu\text{M}$ ). A probability of  $p < 0.05$  was considered significant (one-way ANOVA with Holm-Sidak test).

translocation, SN-50, were examined. Figure 5C shows that ketamine treatment resulted in a significant down-regulation of PSA-NCAM. Application of SN-50 (2.5 $\mu\text{M}$ ) effectively protected neurons from ketamine-induced cell death. Finally, the inactive control peptide for SN-50 (2.5 $\mu\text{M}$ ) was inactive. These data indicate that ketamine-induced neurotoxicity in neonatal monkey frontal cortical cultures manifest primarily as neuronal cell loss.

### *The Effect of NR1 Antisense ODN on the Regulation of the Ketamine-Induced Neural Cell Death*

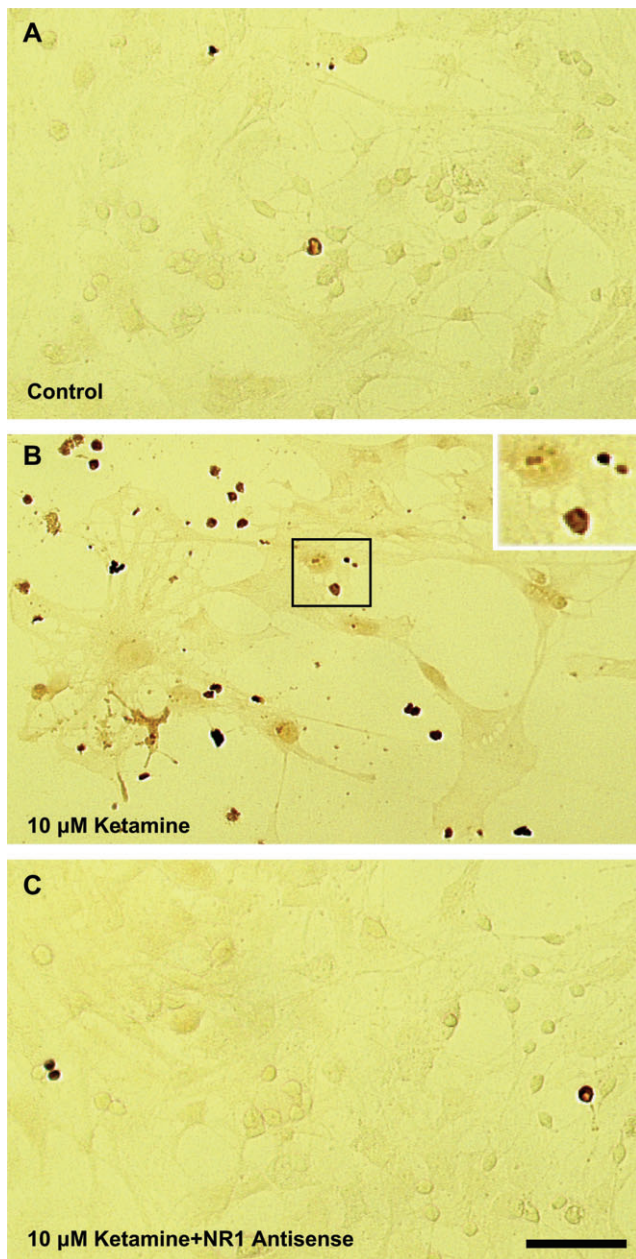
To determine whether enhanced neurodegeneration after ketamine exposure could be due to ketamine-induced upregulation of the NMDA receptor, frontal cortical cultures were treated for 24 h with 10 $\mu\text{M}$  ketamine, and another group was coadministered an NR1 antisense ODN with ketamine. As can be seen in Figures 2a, 2b, and 2c, coadministration of NR1 antisense ODN (2 $\mu\text{M}$ ) blocked the enhanced cell death as indicated by an ELISA and LDH release and prevented the reduction in mitochondrial metabolism of MTT induced by 10 $\mu\text{M}$  ketamine. Importantly, no significant protective effect (Figs. 2a, 2b, and 2c) was observed after the coadministration of NR1 sense ODN with ketamine. In addition, no significant neurotoxic effect was observed in the cultures treated with NR1 antisense ODN alone.

Ketamine-induced cell death was further confirmed by the increased number of TUNEL-positive cells. In the controls (Fig. 6A), only a few TUNEL-positive cells were observed. In cultures treated with 10 $\mu\text{M}$  ketamine, numerous darkly stained TUNEL-positive cells exhibiting typical nuclear condensation and fragmentation (Fig. 6B) were observed. Coadministration of 2 $\mu\text{M}$  NR1 antisense ODN protected neonatal frontal cortical neurons from cell death induced by ketamine (Fig. 6C).

To further define the relationship between altered NMDA receptor levels and enhanced neuronal cell death, neonatal frontal cortical cultures were treated with ketamine (10 $\mu\text{M}$ ) for 24 h with or without NR1 antisense ODN (2 $\mu\text{M}$ ). The NR1 subunit protein level was measured by Western blot analysis and found to be present in both control and ketamine-treated cultures (Fig. 7a). However, ketamine administration produced an upregulation of the NMDA receptor NR1 subunit protein. Quantitative analysis revealed that 10 $\mu\text{M}$  ketamine resulted in a two-fold increase in protein for the NMDA receptor NR1 subunit compared with the control (Fig. 7b).

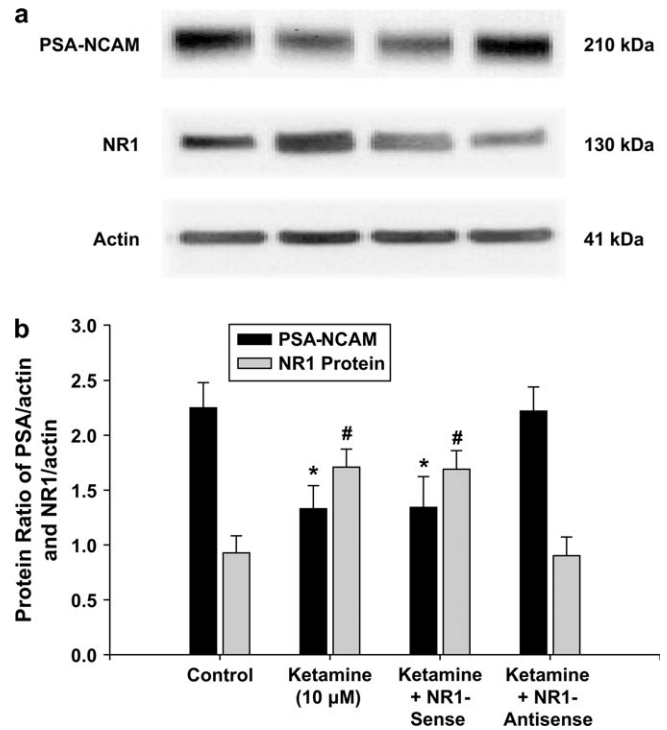
To determine whether the expression of PSA-NCAM, a neuronal-specific marker, correlated with ketamine-induced alterations in NR1 protein, Western blot analysis for PSA-NCAM was also performed. A major protein band at  $\sim 210$  kDa was recognized by the monoclonal antibody (Fig. 7a). Quantitative densitometry revealed that ketamine produced a significant decrease in the PSA-NCAM protein expression compared with the control (Fig. 7b). Coadministration of 10 $\mu\text{M}$  ketamine with NR1 antisense ODN blocked NR1 protein upregulation and the reduction of PSA-NCAM expression induced by ketamine (Figs. 7a and 7b). No similar effect was observed for the cultures coadministered ketamine with NR1 sense ODNs.

The involvement of NMDA receptors in ketamine-induced apoptotic cell death was further supported by the effects of pharmacological blockade of ionotropic glutamate receptors. Cultures were exposed to normal media (control) or ketamine on day 5 for 24 h. After ketamine washout, addition of the



**FIG. 6.** Representative photographs of ketamine-induced neurotoxicity with or without NR1 antisense ODN. Examples of TUNEL labeling of cells are shown in the control culture (A), 10 $\mu$ M ketamine (B), and 10 $\mu$ M ketamine plus 2 $\mu$ M NR1 antisense ODN (C). Cultures were processed for the TUNEL assay 24 h following ketamine washout with serum and glutamate-containing medium. TUNEL-positive cells are clearly more numerous in the 10 $\mu$ M ketamine-treated group (B). Only a few TUNEL-positive cells were observed in the control or in the cultures coadministered NR1 antisense ODN; scale bar, 50  $\mu$ m.

NMDA receptor antagonist, d-APV (100 $\mu$ M for 1 day), to the culture media almost completely blocked the neurotoxicity induced by 10 $\mu$ M ketamine as indicated by the MTT assay (Fig. 8). However, no significant protective effect was observed after the application of either 20 $\mu$ M CNQX (an AMPA and



**FIG. 7.** Western blot analysis of the effect of ketamine and NR1 antisense ODN on the regulation of NMDA receptor NR1 subunit protein and PSA-NCAM expression (a).  $\beta$ -actin was used as a loading control and was not affected by ketamine. The data from three independent experiments were quantified using densitometry and expressed as the ratio of PSA-NCAM to actin and NR1 to actin (b). Statistical comparisons consisted of a one-way ANOVA with the Holm-Sidak test. A probability of  $*p < 0.05$  was considered significant (one-way ANOVA with Holm-Sidak test).

kainate receptor antagonist) or 10 $\mu$ M nifedipine (an antagonist of L-type voltage-sensitive calcium channels). Importantly, no significant neurotoxic effect was observed in cells maintained in defined serum-free media (with absence or low concentration of glutamate) for 24 h after ketamine washout compared with the control (Fig. 8).

## DISCUSSION

Ketamine is a widely used dissociative anesthetic agent. The issue of whether the neurotoxicity observed in young rats has scientific and regulatory relevance for the pediatric use of ketamine relies heavily upon the confirmation of these findings in an appropriate primate model (Haberny *et al.*, 2002).

Primary frontal cortical culture system, established using tissues from PND-3 monkeys, provides a reliable parallel *in vitro* model to assist in evaluating the neurotoxicity of various anesthetics using a minimal number of animals in a short period of time. This system allows the investigation of cellular mechanisms that may be associated with ketamine-induced cell death in a simplified primate system.

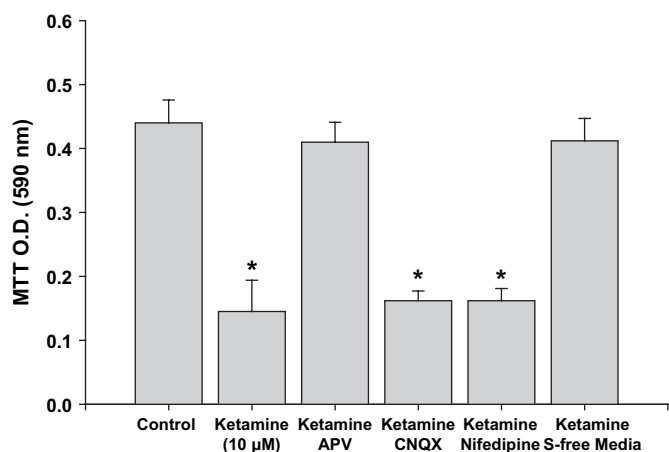


FIG. 8. Effect of pharmacological blockade of ionotropic glutamate receptors on ketamine-induced reduction of mitochondrial metabolism of MTT. Cultures were exposed to normal media (control) and ketamine on day 5 in the culture medium for 24 h. The neurotoxicity was evaluated 24 h after ketamine washout by the MTT assay. After ketamine washout, the addition of APV (100 μM) blocked the neurotoxicity induced by ketamine. No effect was observed with CNQX (20 μM) and nifedipine (10 μM). Values are means  $\pm$  SDs from three independent experiments.

#### Ketamine-Induced Neurotoxicity in the Developing Monkey Brain

Our working hypotheses were that: (1) the exposure of developing brains to ketamine causes selective cell death by a mechanism in which a compensatory upregulation of specific NMDA receptor subunits is involved and (2) ketamine-induced cell death is associated with calcium overload via glutamatergic stimulation of the upregulated NMDA receptors that exceeds the buffering capacity of mitochondria and interferes with electron transport in a manner that results in the production of reactive oxygen species (Scallet *et al.*, 2004; Wang *et al.*, 2003, 2005b).

Overactivation of NMDA receptors is known to kill neurons via a necrotic mechanism characterized by excessive sodium and calcium entry, accompanied by chloride and water entry that leads to cell swelling and death (Rothman *et al.*, 1985). More recently, it has been realized that NMDA receptor activation can also lead to apoptotic cell death (Ankarkona *et al.*, 1995; Wang *et al.*, 2000, 2004). The characteristics of an excitotoxic insult that lead to necrosis or apoptosis are not clear-cut and may depend on the concentration of the glutamate agonist, the duration of the treatment, the receptor subtype activated, and the cell type and its stage of development or maturity (Cheung *et al.*, 1998). Although it was not the intent of the current study to absolutely distinguish between necrosis and apoptosis, the concordance between increased internucleosomal DNA fragments (~42%; mostly apoptotic) and a significant increase in LDH release (~40%; mostly necrotic), coupled with decreased mitochondrial MTT metabolism (75% reduction from total cell viability), following ketamine exposure, suggests that ketamine-induced cell death in monkey cultures has the characteristics of both apoptosis and necrosis.

Meanwhile, in time course studies, addition of 10 μM ketamine results in an approximately 58% loss of viability at 6 h and approximately 75% at 24 h. However, there was no significant difference between control and ketamine exposure at 2 h. We suggest that modest activation (serum and glutamate-containing medium) of upregulated NMDA receptors over a time period of 6–24 h appears to be critical for the production of ketamine-induced apoptosis and necrosis in developing monkey neurons in frontal cortical cultures.

#### NF-κB Signaling in Ketamine-Induced Neuronal Cell Death

The transcription factor NF-κB is known to respond to changes in the redox state of the cytoplasm and has been shown to translocate in response to NMDA-induced cellular stress (Ko *et al.*, 1998). NF-κB is normally sequestered in the cytoplasm, bound to the regulatory protein IκB. In response to a wide range of stimuli including oxidative stress, IκB is phosphorylated on serine residues by the enzyme IκB kinase. The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus.

NF-κB translocation appears to be a necessary step in cell death induced by PCP (McInnis *et al.*, 2002), cyanide, and excitotoxic stimuli (Shou *et al.*, 2000). In this study, ketamine produced a remarkable increase in translocation of NF-κB into the nucleus. The protection against cortical neuronal cell death and decreased PSA-NCAM by a peptide inhibitor of NF-κB translocation, SN-50, suggests that there is a causal relationship between these effects. There is evidence in the literature suggesting that the transcriptional regulation of target genes by NF-κB is tissue specific and possibly gene specific within a given cell type. The ability of SN-50 to prevent ketamine-induced cell death demonstrates that NF-κB is crucial to these processes. However, whether ketamine-induced NF-κB translocation is specifically responsible for apoptotic or necrotic pathways observed in this study is still unknown. Resolution of this question will require additional experiments.

The sialylation state of NCAM (PSA-NCAM) is controlled by developmentally regulated Golgi sialyltransferase activity (Breen and Regan, 1988). This transferase activity is Ca<sup>2+</sup> dependent (Bruses and Rutishauser, 1998), and this may account for its regulation by NMDA receptors (Butler *et al.*, 1999; Wang *et al.*, 1996). The regulation of PSA-NCAM expression by NMDAergic activity plays a critical role in neuroplasticity during development, particularly in NCAM-mediated cell-cell interactions and synapse formation (Szele *et al.*, 1994).

In this study, treatment of frontal cortical cultures from the developing monkey with ketamine caused a substantial decrease in mitochondrial metabolism of MTT along with a concomitant decrease in PSA-NCAM staining. The decrease in PSA-NCAM corresponded to an approximately 40% decrease in PSA-NCAM immunoreactivity. This decrease could be the direct result of local NMDA receptor blockade (and subsequent reduction in Ca<sup>2+</sup>-regulated polysialyl transferase



activity) or the indirect result of cortical neuronal loss. The fact that SN-50 dose dependently blocked cortical neuronal cell death, as well as the loss of PSA-NCAM immunoreactivity in the culture, argues for the latter mechanism. Future experiments using N-butanoyl-mannosamine to inhibit polysialyl transferase or endoneuramidase N to selectively cleave PSA chains may be able to further address this possibility.

#### *Regulation of NMDA Receptor in Ketamine-Induced Neuronal Cell Death*

Of particular interest to the data at hand are the possible mechanisms by which ketamine treatment could upregulate NMDA receptors. Surprisingly, there is not an abundance of literature concerning this issue, but recently it has been demonstrated that the distal region of the NR1 promoter contains an active NF- $\kappa$ B site, which is developmentally regulated and appears to bind Sp3/Sp1 somewhat better than the NF- $\kappa$ B subunits (Liu *et al.*, 2004). The NMDA receptor NR1 subunit is widely distributed throughout the brain and is the fundamental subunit necessary for NMDA channel function. This study demonstrated that ketamine produces a significant upregulation in NR1 protein expression. This result is consistent with literature demonstrating that treatment with NMDA antagonists produces an upregulation of the NMDA receptor complex as measured by an increase in  $B_{\max}$  of NMDA receptor binding sites (McDonald *et al.*, 1990; Williams *et al.*, 1992). Chronic treatment with ethanol, another noncompetitive NMDA antagonist (Lovinger *et al.*, 1989), has also been shown to upregulate NMDA receptor number and function both *in vitro* and *in vivo* (Grant *et al.*, 1990; Hu *et al.*, 1996; Trevisan *et al.*, 1994). In addition, chronic ethanol stabilizes NR1 mRNA in fetal cortical neurons in culture (Kumari and Ticku, 1998).

In previous rodent studies, we observed that administration of PCP, a noncompetitive NMDA antagonist, enhanced NMDA receptor expression and function (Wang *et al.*, 1999, 2000). In this study, ketamine produced an upregulation of NMDA receptor NR1 subunit protein that was accompanied by enhanced neuronal loss (decreased expression of PSA-NCAM). The effect of ketamine to enhance neurodegeneration could be the result of a ketamine-induced upregulation (compensatory) of NMDA receptors for glutamate (serum and glutamate-containing medium). In fact, this hypothesis was supported from cells kept in defined serum-free media (absence or low concentration of glutamate) for 24 h after ketamine washout. No significant effects as measured by MTT metabolism were observed in this group compared with the control.

To test whether the administration of antisense ODNs targeted to the NR1 NMDA receptor subunit blocks steady-state protein levels, an antisense ODN-targeting exon was used. Coadministration of NR1 antisense ODN was able to almost completely block the neuronal cell death induced by ketamine. Our data indicate that ketamine remarkably upregulated NMDA receptor NR1 subunit protein. Coadministration of antisense

ODN specifically prevented NR1 upregulation and blocked the reduction of PSA-NCAM expression induced by ketamine.

Neuronal cells in frontal cortical cultures are known to express other  $\text{Ca}^{2+}$  channels such as voltage-dependent  $\text{Ca}^{2+}$  channels and non-NMDA, AMPA/kainate receptors that have been shown to be  $\text{Ca}^{2+}$  permeable (Nakanishi, 1992). To further confirm that compensatory upregulation of NR1 subunits and the subsequent increase in NMDA receptor function are specifically responsible for ketamine-induced cell death, pharmacological studies were performed. After ketamine washout, application of the AMPA/kainate receptor antagonist, CNQX, had only a small (negligible) effect as compared to the strong protective effect obtained with the selective NMDA receptor antagonist, d-APV (100  $\mu\text{M}$  for 1 day). Similarly, nifedipine, an antagonist of L-type voltage-sensitive calcium channels, did not produce a significant protective effect. These results strongly suggest that enhanced neurodegeneration observed in the frontal cortical culture is associated with the upregulation of NMDA receptors.

Taken together, these experiments demonstrate that ketamine-induced neuronal cell death in monkey frontal cortical cultures appears to be both apoptotic and necrotic in nature. Ketamine produces an apparent increase in the NMDA receptor NR1 subunit expression. Coadministration of NR1 antisense ODNs specifically prevents synthesis of NMDA receptor NR1 protein and subsequently blocks the neuronal loss induced by ketamine.

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