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Ketamine Activates Cell Cycle Signaling and Apoptosis in the Neonatal Rat Brain

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ABSTRACT

Background: Prolonged exposure to ketamine results in accelerated neurodegeneration and neurocognitive deficits in the neonatal rats. Experimental models of neurodegeneration have implicated reentry of postmitotic neurons into the cell cycle, leading to cell death. The authors hypothesize that the ketamine-induced neuroapoptosis is partially due to aberrant cycle cell reentry. To explore this hypothesis, the authors characterized the effect of ketamine on the cell cycle signaling pathway in the developing rodent brain *in vivo* and *in vitro*.

Methods: Postnatal day 7 rat pups and primary neurons were used for the experiments. Each rat pup received five intraperitoneal doses of either saline or ketamine (5, 10, and 20 mg/kg/dose) at 90-min intervals over 6 h. Primary neurons were exposed to varying concentrations of ketamine to determine the dose and duration effects. The expression of cell cycle proteins (cyclin D1, cyclin-dependent kinase 4, and E2F1), Bcl2-interacting mediator of cell death (Bim), and activated caspase-3 was determined. The effect of cyclin D1 knockdown by small interfering RNA was also examined in primary neurons incubated in ketamine.

Results: Ketamine mediated a dose- and time-dependent increase in expression of cell cycle proteins and activated caspase-3. Cyclin D1, cyclin-dependent kinase 4, E2F1, Bim, and cleaved caspase-3 expression increased at 12 h and peaked at 24 h *in vitro*. Knockdown of cyclin D1 by small interfering RNA attenuated Bim and cleaved caspase-3 expression.

Conclusion: These findings support a model in which ketamine induces aberrant cell cycle reentry, leading to apoptotic cell death in the developing rat brain.

What We Already Know about This Topic

- ❖ Ketamine enhances cell death in the brains and cognitive deficits of young animals
- ❖ Neuronal entry into the proliferative cell cycle leads to cell death and may be a general cause of neurodegeneration

What This Article Tells Us That Is New

- ❖ In 7-day-old rats, ketamine increased expression of cell cycle proteins in neurons of the brain, and using small interfering RNA against these proteins reduced the markers of cell death from ketamine exposure
- ❖ Ketamine may enhance the brain cell death by causing neurons to enter the cell cycle

KETAMINE, an *N*-methyl-D-aspartate receptor (NMDA-R) antagonist is routinely administered to induce anesthesia and alleviate procedural pain in pediatric patients. However, recent preclinical studies in the rodents clearly demonstrate that prolonged exposure to ketamine results in widespread neurodegeneration and long-term neurocognitive deficits.[1–4] Given the ubiquitous use of this drug in infants and children, the specter of ketamine-induced neurodegeneration has emerged as a public health concern.[5]

The cell cycle is the biologic process by which the eukaryotic cells proliferate. Although it is vital for many cell types during early development and in cells that continue to proliferate, pathologic reentry into the cell cycle leads to uncontrolled proliferation and tumorigenesis. However, terminally differentiated neurons, characterized as postmitotic, are unable to proliferate. For postmitotic neurons, activation of cell cycle results in aborted progression through the cell cycle, leading to apoptotic cell death rather than tumorigenesis.[6,7] The protooncogene cyclin D1, an important regulator of cell

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cycle entry, has been proposed as the trigger for the apoptotic cell death pathway during both development and neurodegeneration.[8] Activation of cyclin D1 initiates aberrant reentry into the cell cycle by binding to cyclin-dependent kinase 4 (cdk4).[9] This complex phosphorylates downstream transcription factor E2F1. In postmitotic neurons, phosphorylation of E2F1 has the potential to regulate transcription of the Bcl2-interacting mediator of cell death (Bim). Bim activates caspase-3, resulting in apoptotic cell death.

Experimental models of neurodegeneration have implicated these cell cycle–related proteins and cell cycle reentry as a potential mechanism for apoptotic cell death.[10] This possibility has not been investigated in ketamine-induced neurodegeneration. We hypothesized that ketamine induces cyclin D1-mediated cycle cell reentry, leading to neuronal cell death, and knockdown of cyclin D1 would attenuate this response. To explore this hypothesis, we characterized the effect of ketamine on the cell cycle pathway in the developing rat brain *in vivo* and *in vitro*.

Materials and Methods

Animals

With the approval of the Investigational Review Board and adherence to the *Guide for the Care and Use of Laboratory Animals*,[11] all experiments used Sprague-Dawley postnatal day 7 (P7) rat pups and timed pregnant dams (Charles River Laboratories, Wilmington, MA).

In Vivo *Experiments*

The experiments were conducted in a temperature-controlled acrylic container maintained at 36.7°C. To minimize handling of the pups, direct core temperature measurements were not obtained. However, similar conditions resulted in core body temperatures between 36.5° and 37.5°C.[12] Each rat pup received five intraperitoneal injections (10 ml/kg each) of either ketamine (5, 10, or 20 mg/kg/injection) or vehicle (saline) at 90-min intervals over 6 h. This dosing regimen is similar to that used in previous investigations using a similar experimental paradigm that yields a ketamine plasma concentration of 5.80 ± 3.10 μg/ml and brain concentration of 2.65 ± 1.60 μg/g.[13] The rat pups were kept away from their dam and visually monitored for respiratory effort and activity. To examine the intermediate cell signaling changes, rat pups were euthanized with pentobarbital (100 mg/kg intraperitoneally) 6 h after the initial injection. Saline- and ketamine-treated rat pups (n = 6 each for the control and 20 mg/kg groups) were perfused transcardially with saline followed by 4% paraformaldehyde. The brains were embedded in paraffin for histologic processing. A second cohort of saline- and ketamine-treated rat pups (n = 6 each for control and three dosing groups) were subjected to the same experimental paradigm, and the extracted brains were immediately frozen in liquid nitrogen and processed for protein analysis.

In Vitro *Experiments*

Cortical neuron cultures were prepared from embryonic day 18 Sprague-Dawley rat fetuses. In brief, the pregnant dams were euthanized with carbon dioxide in a chamber, and the fetuses were removed quickly, placed on ice, and decapitated. The cortices of the fetal brain were dissected rapidly in ice-cold Hank's balance salt solution. The tissue was dissociated with 0.25% trypsin–ethylenediamine-tetraacetic acid in Hank's balance salt solution for 30 min and centrifuged. Cortical neurons were resuspended in Neurobasal medium (Invitrogen, Carlsbad, CA) containing 1× B27 supplement, 500 μM glutamine, and 10 μg/ml gentamycin (Invitrogen). To assure the immature state of the primary neurons in the *in vitro* experiments, the cells were plated and cultured for 3 days *in vitro* at 37°C and 5% carbon dioxide. Racemic ketamine (K2753; Sigma, St. Louis, MO) resuspended in sterile water was added directly into medium at various concentrations for different time periods, as indicated, then washed out with phosphate-buffered saline. Cells were then harvested for cell viability assay, immunostaining, or Western blotting.

To determine the percentage of neurons present, cortical cultures were fixed with 4% paraformaldyhyde for 15 min and immunostained with the neuron-specific mouse Tuj1 antibody (1:300, Covance, Princeton, NJ) overnight, followed by goat antimouse Cy3 (1:300, Jackson Immunoresearch, West Grove, PA) secondary antibody. In addition, nuclei were stained with 4′-6-diamidino-2-phenylindole (1:10,000, Sigma). The neuronal percentage was determined by manually counting the number of double-stained cells (visualized as pink immunofluorescence) per optical field and the number of cells stained with 4′-6-diamidino-2-phenylindole alone. Ninety percentage of the cells in our cortical cultures were neurons, evidenced by Tuj1 staining (fig. 1). Most Tuj-1-immunoreactive cells had dendritic spines as visualized by fluorescence microscopy (fig. 1A).

Western Blot Analysis

To measure the effect of ketamine on the cell cycle proteins and caspases of the intrinsic apoptotic cell death pathway, total protein was isolated from primary neurons using cell lysis buffer containing protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (Sigma), or extracted from flash-frozen brain tissue with radioimmunoprecipitation assay buffer (Sigma) containing protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride. Protein concentrations were measured by DC protein assay (Bio-Rad, Hercules, CA). The equal amounts of protein were boiled in sodium dodecyl sulfate loading buffer (Bio-Rad), then resolved on 8–12% polyacrylamide denaturing gels, and transferred to nitrocellulose (Bio-Rad). Antibodies used for Western blotting included rabbit antibodies to cleaved caspase-9 and caspase-3, cyclin D1, cdk4, E2F1, E2F4, Bim, and β-actin (1:2,000; Cell Signaling, Danvers, MA). The blots were washed, and the species-matched peroxidase-conjugated secondary antibody was added. Labeled bands from each blot were detected by enhanced chemiluminescence for visualization and quantitation (Thermo Scientific, Waltham, MA).

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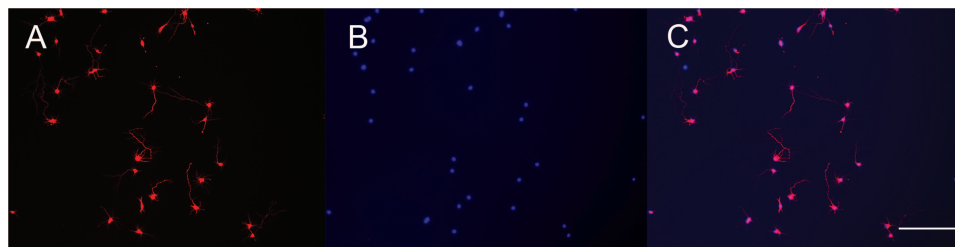

Fig. 1. The cortical cells in culture are primarily neurons. The cortical cells were dissociated and cultured for 3 days before fixation and immunohistochemistry. These cells were imaged with fluorescence microscopy. (*A*) Neurons were identified with Tuj1 antibody (*red*). (*B*) Nuclear-specific 4′-6-diamidino-2-phenylindole stain (*blue*) labeled all cells. (*C*) Merged image: counting revealed that 90% of cultured cells are neurons (*pink*; scale bar = 200 μm).

Caspase-3 Immunohistochemistry

Apoptotic neurons were identified by immunohistochemical detection of cleaved caspase-3, the terminal enzyme of the apoptotic cell death pathway.

In Vivo. Brain sections (5 μm) were incubated with cleaved caspase-3 antibodies in a 1:100 dilution overnight, followed by incubation with polyclonal goat antirabbit secondary Immunoglobulin G antibody (Dako, Carpinteria, CA) and subsequently conjugated with horseradish peroxidase for visualization. Only the cortex and thalamus were examined, because previous investigators have demonstrated the greatest effect in these regions.[3] Neurons that were stained brown (cleaved caspase-3) were counted in a blinded manner at 400× magnification. The focal plane was moved throughout the depth of the slice to allow counting of stained cells in all planes. The number of positive cells was calculated using the stereologic dissector method. Results from six randomly selected sampling volumes were averaged for each brain region (cortex and thalamus).

In Vitro. Primary neurons (3 days *in vitro*) were prepared on cover slips in 24-well plates, treated with ketamine for 6–48 h, and then fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized with 0.01% Triton X-100 in phosphate-buffered saline for 30 s, blocked with 1% bovine serum albumin for 30 min, incubated with rabbit anticleaved caspase-3 antibody (1:500; Cell Signaling) overnight at 4°C, followed by goat antirabbit antibody (1:2000; Cell Signaling) for 1 h at room temperature, and stained with Fast Red (Millipore, Billerica, MA).

To determine the cell type that expressed cleaved caspase-3, a cohort of brain sections and cortical cells (n = 3) were incubated with rabbit anticleaved caspase-3 (1:2500; Cell Signaling) and neuron-specific mouse anti-NeuN (1:100; Abcam, Cambridge, MA) antibodies overnight. This was followed by incubating the tissue for 1 h with the following secondary antibodies: Cy3-conjugate donkey antirabbit (Jackson ImmunoResearch) and Streptavidin-Alexafluor 488 conjugate (Invitrogen). All secondary antisera were diluted to 1:200. Both primary and secondary antisera were diluted in 0.1 M phosphate buffer with normal saline, 0.3% Triton x-100, 0.04% bovine serum albumin, and 0.1% sodium azide. The processed tissue was rinsed in 0.1 M phosphate buffer in saline solution before mounting on slides from a 0.05 M phosphate buffer solution. After drying, mounted sections were covered with 90% glycerol. Finally, the slides or fixed cultures were imaged with fluorescent microscope (Olympus IX81; Olympus, Center Valley, PA) equipped with a camera and digital microscopy software (Slidebook version 4.2, Olympus), and the cell counts were performed as described previously.

To determine the specific role of cyclin D1 on ketamine-induced neuroapoptosis, primary neurons resuspended in reduced serum media (OptiMEM I; Invitrogen) were transfected with small interfering RNA (siRNA) against cyclin D1 (SC-156083; Santa Cruz Biotechnology, Santa Cruz, CA) or scramble-control siRNA (SC-37007; Santa Cruz Biotechnology) using a lipid-based transfection reagent (Lipofectamine 2000; Invitrogen) for 6 h. The neurons were resuspended in Neurobasal medium containing 1× B27 supplement, 500 μM glutamine, and 10 μg/ml gentamycin for 24 h. The cells were then incubated in 10 μM ketamine for 24 h. The primary neurons were then subjected to immunoblotting as described earlier.

To examine the effect of ketamine on cell viability, we plated primary neurons in 96-well plates, cultured for 3 days, then treated with ketamine for 6 or 48 h. Cell viability was determined using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Millipore) following the manufacturer's protocol.

Statistical Analysis

The changes in cell viability, caspase-3 activation, and cell cycle protein levels were presented as percentages of control values. The mean and SE for the control and ketamine groups were compared using two-tailed unpaired *t* test and one-way ANOVA followed by *post hoc* Dunnett's test for comparisons with control values. Significance was set at *P* values less than 0.05.

Results

Ketamine Induces Activation of the Cell Cycle Proteins Cyclin D1 and cdk4

We used a previously established *in vivo* model of anesthetic-induced neurodegeneration to assess the effect of ketamine on cell cycle proteins in the developing rat brain.[2,3] Each rat pup received five intraperitoneal injections (10 ml/kg each) of either ketamine (5, 10, or 20 mg/kg/injection) or vehicle (saline) at 90-min intervals

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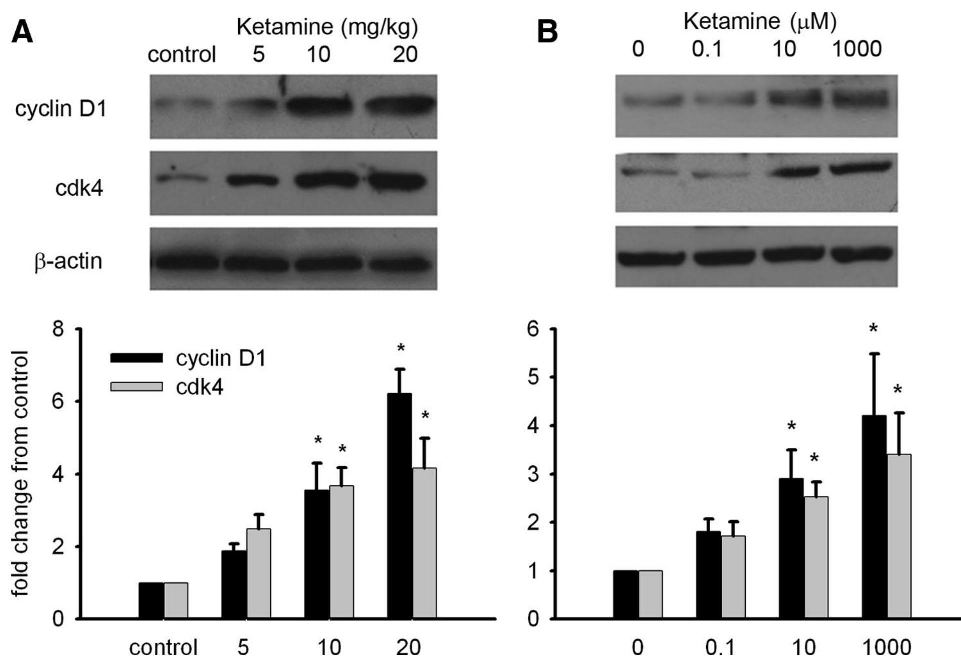

Fig. 2. Ketamine increases cyclin D1 and cyclin-dependent kinase 4 (cdk4) expression. (*A*) Western blot of the brain extracts from postnatal day 7 rats treated with repeated injections of saline or ketamine show a dose-dependent increase in both cyclin D1 and cdk4 (n = 3, * $P < 0.05$). (*B*) Western blot of protein lysates from primary neurons exposed to ketamine at different concentrations demonstrate a similar pattern (n = 4, * $P < 0.05$). Data are presented as mean ± standard error.

over 6 h. Ketamine produced dose-dependent increases in cyclin D1 and cdk4 expression in the P7 rat brain (fig. 2A). Western blots of brain lysates from saline- and ketamine-treated P7 rat pups revealed a cyclin D1 immunoreactive band at 34 kD, which increased in concentration after ketamine treatment. By this measure, cyclin D1 increased 3.5- and 6.2-fold over control values for the 10 and 20 mg/kg ketamine doses, respectively ($P < 0.05$). Similarly, the protein concentration of the cdk4 immunoreactive band at 30 kD increased 3.6-fold ($P < 0.05$) and 4.1-fold ($P < 0.05$) after the administration of 10 and 20 mg/kg ketamine, respectively. The increases in cyclin D1 and cdk4 after the administration of 5 mg/kg ketamine were not significant.

In vitro, ketamine concentrations ranging from 0.1 to 1,000 μM mediated a dose-dependent increase in cyclin D1 and cdk4 protein levels. Primary neurons incubated in 10 μM ketamine for 24 h exhibited a 3.6-fold increase in cyclin D1 expression and a 2.5-fold increase in cdk4 expression compared with control (fig. 2B).

Ketamine Induces Activation of E2F1 and Bim

Exposure to ketamine also induced a dose-dependent increase in expression of E2F1 and Bim protein in P7 rat brain (fig. 3A). Antibodies to E2F1 and Bim recognized bands at 50 and 20 kD, respectively, on Western blots of P7 brain extracts. Basal levels of E2F1 protein were detected in the control group, and the administration of ketamine resulted in 2.8- and 3.3-fold increases for the 10 and 20 mg/kg groups, respectively ($P < 0.05$). E2F4 levels were not affected by increasing doses of ketamine *in vivo*. Immunoblotting also revealed 3- and 5-fold increases in Bim protein induced by 10 and 20 mg/kg ketamine, respectively.

Primary neurons exposed to ketamine also demonstrated a dose-dependent increase in E2F1 and Bim levels (fig. 3B). Incubation with 10 μM ketamine for 24 h produced a 3.6-fold increase in E2F1 expression and a 2.5-fold increase in Bim expression.

Ketamine Induces Caspase-3 Activation

Previous studies have established caspase-3 activation as a marker of impending apoptotic cell death in this experimental model of anesthetic-induced neurodegeneration.[3,14] To investigate whether this neuronal death involves caspase-3, we examined the appearance of cleaved caspase-3 in the brain slices from rats receiving 20 mg/kg ketamine every 90 min over 6 h. P7 rat pups were euthanized at 6 h after the initial dose, and the brains were removed and sectioned for analysis. Light microscopy of the brain sections from ketamine-treated rat pups exhibited a 5-fold increase in cleaved caspase-3 positive cells compared with the saline-treated controls in both the cortex and the thalamus (fig. 4A–C). Immunofluorescence microscopy of the brain sections stained with antibodies to cleaved caspase-3 (red) and NeuN, a neuron-specific nuclear protein (green) antibody, demonstrated that most of the cleaved caspase-3 positive cells were neurons (orange-yellow) (fig. 4D).

Primary neurons exposed to ketamine exhibited a dose-dependent increase in cleaved caspase-3 protein (fig. 5A and B). Double staining of these cells with cleaved caspase-3 and NeuN antibodies revealed a significant increase in cleaved

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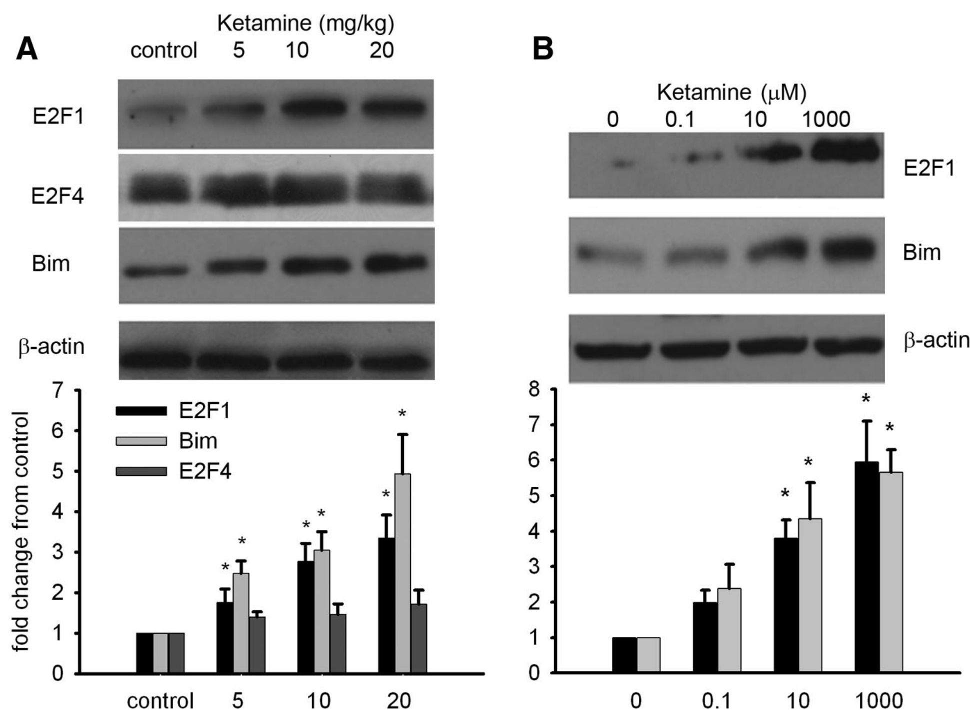

Fig. 3. Ketamine increases E2F1 and proapoptotic Bcl2-interacting mediator of cell death (Bim). (*A*) Western blot of the brain extracts from postnatal day 7 rats treated with repeated injections of saline or ketamine. Ketamine produced a dose-dependent increase in E2F1 and Bim. E2F4 did not change (n = 3, * $P < 0.05$). (*B*) Western blot of protein lysates from primary neurons also revealed a dose-dependent increase in E2F1 and Bim concentrations (n = 4, * $P < 0.05$). Data are presented as mean ± standard error.

caspase-3 in primary neurons treated with ketamine when compared with control (fig. 5C–F). These data show that prolonged exposure to high concentrations of ketamine results in a caspase-3–mediated cell death.

To verify that the intrinsic apoptotic pathway was activated in ketamine-induced neuroapoptosis, we measured the levels of cleaved caspase-9 and caspase-3 protein in the brain lysates from P7 rat pups treated with incremental doses of ketamine for 6 h. Consistent with previous reports, we observed a dose-dependent increase in the intensity of the cleaved caspase-9 and caspase-3 (fig. 6).

Time Course of Ketamine-induced Activation of Cell Cycle Proteins

The increases in cyclin D1 and cdk4 protein levels reached significance after 12 h of 10 μM ketamine exposure and remained increased at 48 h. The increases in Bim expression were also time-dependent and peaked at 24 h. There was some baseline expression of cleaved caspase-3 in the untreated cells, indicating a low level of ongoing apoptosis. However, cleaved caspase-3 levels increased significantly and peaked similarly at 24 to 48 h (fig. 7A and B).

siRNA Knockdown of Cyclin D1 Attenuates Ketamine-induced Increases in Bim and Cleaved Caspase-3

Ketamine increased levels of cyclin D1 and its downstream cell cycle proteins in a dose- and time-dependent manner. Therefore, siRNA directed against cyclin D1 should decrease caspase-3 activation. Cyclin D1 is not constitutively expressed, and there was no change in cyclin D1 expression in the scramble-siRNA and cyclin D1-siRNA transfected cells. However, ketamine-treated primary neurons transfected

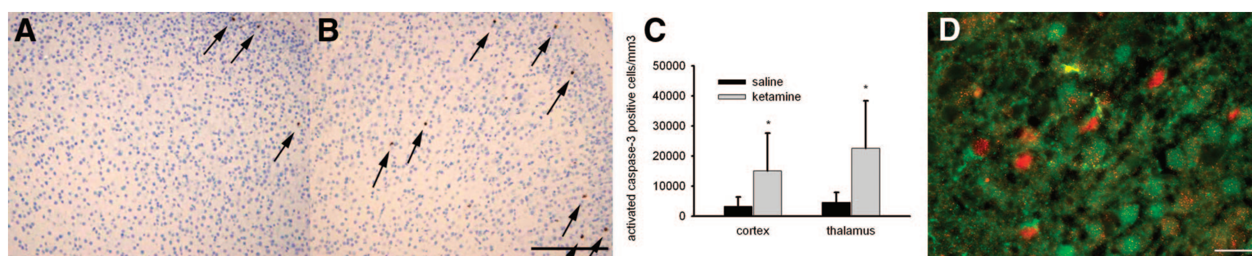

Fig. 4. Ketamine induces neuronal caspase-3 activation *in vivo*. Immunohistochemistry with antibody to cleaved caspase-3 (*brown*) (*arrows* identify cleaved caspase-3 positive cells) on cortical sections from postnatal day 7 (P7) rat pups receiving either saline (*A*) or 20 mg/kg every 90 min for 6 h ketamine (*B*) (scale bar = 200 μm). (*C*) Quantitation of cleaved caspase-3-immunoreactive cells in P7 cortex and thalamus demonstrated increased cleaved caspase-3 positive cells in the ketamine-treated animals. Data are presented as mean ± standard error compared with saline (n = 4, * $P < 0.05$). (*D*) Double staining with antibodies to cleaved caspase-3 (*red*) and NeuN antibodies, neuron-specific nuclear protein (*green*) antibodies, demonstrated that most of the cleaved caspase-3 positive cells were neurons (scale bar = 20 μm).

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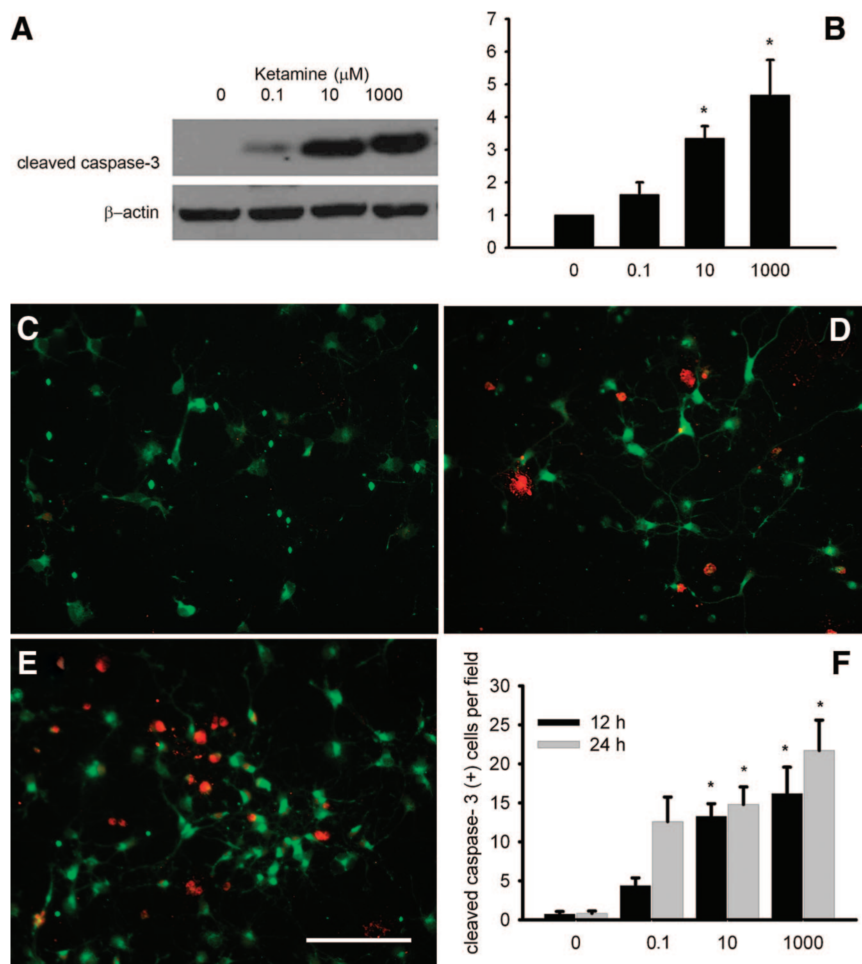

Fig. 5. Ketamine induces caspase-3 activation in primary neurons. Primary neurons were incubated in ketamine (0, 0.1, 10, and 1000 μM) for 12 or 24 h. (*A*) Western blot from primary neurons exposed to ketamine at different concentrations shows a dose-dependent increase in cleaved caspase-3. (*B*) Quantitation of immunoreactive band densities normalized to β-actin and confirmed the significant increases of cleaved caspase-3 when compared with control (n = 4, * $P < 0.05$). Immunofluorescence microscopic of primary neurons stained with antibodies to cleaved caspase-3 (*red*) and NeuN (*green*) antibodies demonstrated that most of the cleaved caspase-3 positive cells were neurons. Primary neurons incubated for 24 h in (*C*) control media, (*D*) ketamine 0.1 μM, and (*E*) ketamine 10 μM. (F) Quantitation of cleaved caspase-3-immunoreactivity in primary neurons incubated ketamine for 12 or 24 h confirmed a dose-dependent effect of ketamine on neuroapoptosis (n = 4, * $P < 0.05$, compared with the control group). Data are presented as mean ± standard error (scale bar = 20 μm).

with cyclin D1-siRNA had a 4-fold decrease in cyclin D1 expression when compared with naïve cells incubated in ketamine (fig. 8).

Ketamine-induced Neurotoxicity Is Dose and Duration Dependent

Exposure to 10 μM ketamine for 48 h induced an abundance of cleaved caspase-3 positive cells in culture when compared with control (fig. 9A and B). We used a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to examine the effect of ketamine on neuronal viability *in vitro*. Primary neurons were incubated in ketamine for 6 or 48 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed a dose- and time-dependent decrease in cell viability (fig. 9C). Neurons treated with ketamine for 6 h did not exhibit significantly reduced cell viability. However, neurons treated for 48 h demonstrated a dose-dependent decrease in cell viability ($P < 0.05$).

Discussion

This study demonstrates that prolonged exposure to ketamine increases expression of regulatory proteins of the cell cycle machinery, which in turn triggers the intrinsic apoptotic pathway. As highly differentiated postmitotic cells, neurons do not normally enter the cell cycle. However, several lines of evidence have implicated aberrant cell cycle reentry as one of many pathologic pathways involved in neurodegenerative diseases and experimental paradigms, such as trophic withdrawal, traumatic brain injury, stroke, and Alzheimer Disease.[7] On the basis of these experimental models of neurodegeneration, our data support the notion that aberrant cycle cell reentry is associated with prolonged exposure to ketamine leading to neuroapoptosis. These regulatory proteins include cyclin D1 and its downstream effectors, cdk-4, E2F1, and Bim. Activation of the initiator caspase-9 and executioner caspase-3 were also increased, indicating activa-

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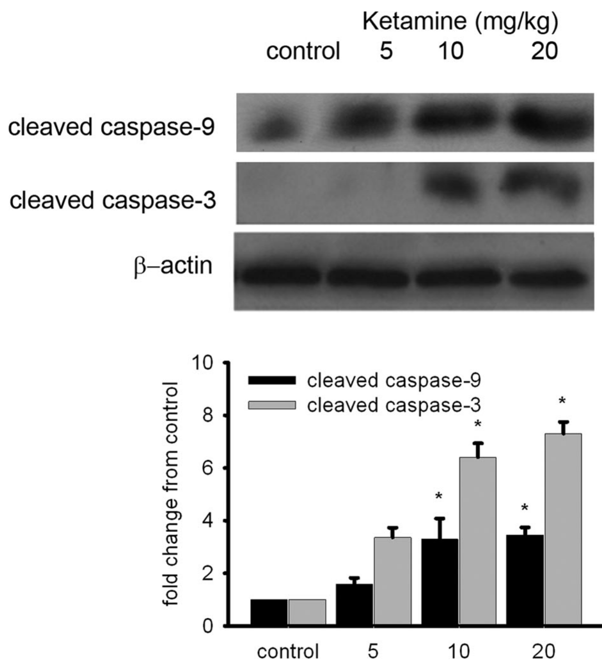

Fig. 6. The intrinsic apoptotic pathway was activated in ketamine-induced neuroapoptosis. Western blot from postnatal day 7 brain protein lysates demonstrate dose-dependent increases in cleaved caspase-9 and -3 with exposure to ketamine (n = 3, * $P < 0.05$). Data are presented as mean ± standard error.

tion of the intrinsic apoptotic pathway. Primary neurons incubated in ketamine developed similar pattern of cell cycle protein and caspase-3 activation. Knockdown of cyclin D1 by siRNA attenuated its downstream effector Bim and cleaved caspase-3. These findings suggest that ketamine activates the regulatory proteins of the cell cycle pathway that serves as another trigger for the neuronal apoptosis in neonatal rat pups.

Cell cycle proteins are active during neurogenesis when the developing brain undergoes both proliferation and apoptosis. Cyclin D1 is a regulatory oncogene that initiates cell cycle entry in a variety of physiologic and pathologic biologic processes.[15] This is especially true during the proliferative phase of neurogenesis that occurs during the embryonic period in rodents, when apoptosis occurs in more than 50% of proliferating cells in the newborn rat brain.[6] Administration of the NMDA-R antagonist MK-801 to P7 rat pups impaired cell proliferation in these areas and significantly increased apoptosis of nonproliferating cells, including postmitotic neurons and glia.[16] By P7, neurogenesis is declining and synaptogenesis peaks.[17] Consistent with this report, we found that ketamine induces significant activation of caspase-3 in P7 cortex and thalamus, regions that are primarily postmitotic. Although we did not distinguish between proliferating and differentiated neuronal populations in our *in vivo* experiments, the neurons in our *in vitro* experiments were derived from embryonic day 18 cortices, an area with minimal progenitor cells, and, thus, were primarily postmitotic. In addition, the presence of dendritic arborization suggested that these were primarily postmitotic neurons (fig. 1).

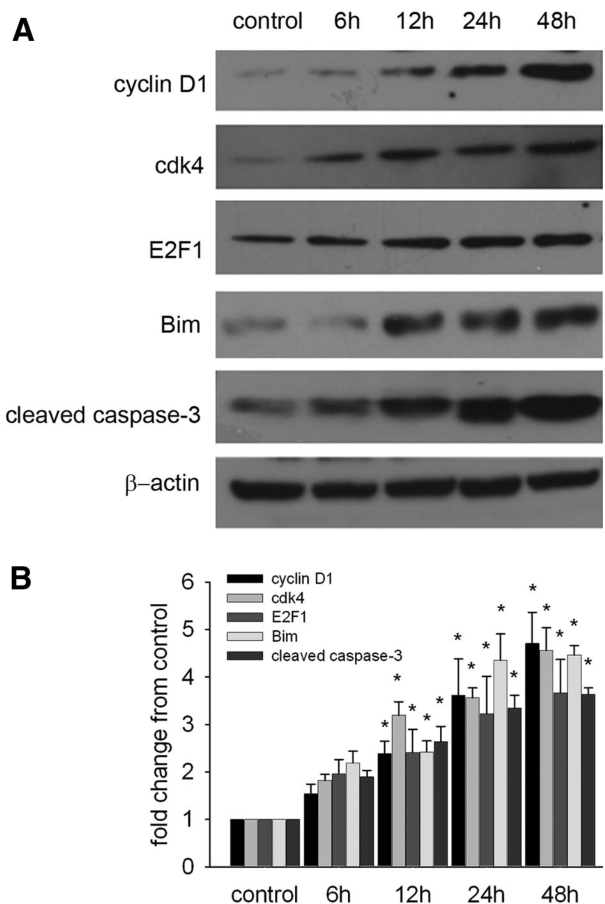

Fig. 7. Time course of ketamine-induced activation of cell cycle proteins. (*A*) Western blots of protein lysates from primary neurons exposed to ketamine (10 μM) for different incubation periods demonstrate a coordinated time-dependent increase in cell cycle–related proteins. (*B*) Quantitation of the immunoblots confirm the significant increases in immunoreactive band densities (n = 4, * $P < 0.05$, compared with the control group). Data are presented as mean ± standard error. Bim = Bcl2-interacting mediator of cell death; cdk4 = cyclin-dependent kinase 4.

Cell cycle reentry has been proposed as one of many neuronal death pathways active during development and neurodegenerative conditions.[7] Cyclin D1 is the regulatory mediator of mitogeneic signals to cell cycle machinery. We demonstrate that ketamine increased cyclin D1 expression leading to up-regulation of downstream cell cycle proteins and ultimately resulting in casapse-3 activation and neuronal apoptosis. Our data show that ketamine produces a dose- and time-dependent increase in cyclin D1 concentration that mirrored a rise in its catalytic partner cdk4. Cyclin D1 activation was detected at 6 h and peaked by 24–48 h. This was followed by increasing expression of cdk4, E2F1, and Bim. Cyclin D1/cdk4 complex phosphorylates retinoblastoma protein, which binds to E2F1. The cyclin D1/cdk4 complex also phosphorylates p130, which binds to E2F4. Both E2F1 and E2F4 are transcriptional factors that have opposing effects on cell cycle progression.[18] E2F1 is transcriptional activator that promotes cell cycle progression. E2F4 has the opposite effect and is a cell cycle repressor. The current study

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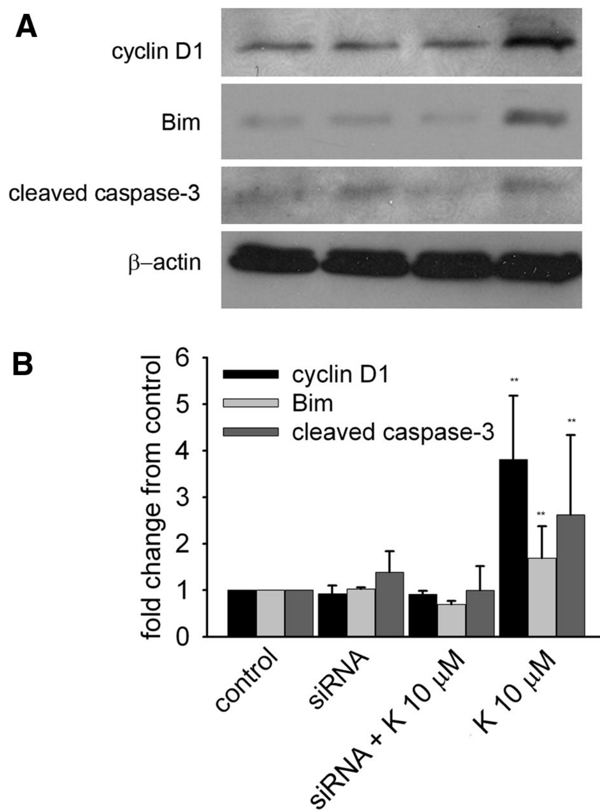

Fig. 8. Small interfering RNA (siRNA) knockdown of cyclin D1 attenuates ketamine-induced increases in Bcl2-interacting mediator of cell death (Bim) and cleaved caspase-3. (*A*) Primary neurons were transfected with cyclin D1-siRNA (siRNA) or scrambled control-siRNA (control) for 6 h and exposed to 10 μM ketamine or control medium for 24 h. Knockdown of cyclin D1 by siRNA resulted in decreased expression of Bim and cleaved caspase-3. (*B*) Quantitation of immunoreactive band densities normalized to β-actin confirm the attenuation of Bim and cleaved caspase-3 in ketamine-treated primary neurons transfected with cyclin D1-siRNA (n = 3, ** $P < 0.05$, compared with the control group). Dataare presented as mean ± standard error. K = ketamine.

shows that ketamine induces a dose- and time-dependent increase in E2F1 concentration. However, E2F4 expression did not change. This interaction favors cell cycle progression and ultimately apoptosis in postmitotic neurons. A downstream function of E2F1 includes induction of the transcriptional factors B- and C-myc, which in turn induces Bim expression.[19] These observations demonstrate that Bim is a direct downstream target of cyclin D1/cdk-4–mediated cell cycle progression that leads to neuronal apoptosis. Parallel signaling pathways such as c-Jun and FoxO can also induce Bim expression and neuronal apoptosis.[20] In the current study, we show the ketamine also increases Bim concentration in the dose- and time-dependent manner. Low levels of cleaved caspase-3 were detected at control and at 6 h, gradually increased at 12 h and peaked at 48 h. This delayed pattern is consistent with ongoing physiologic apoptosis early in this time course and accentuated apoptosis corresponding to increases in cell cycle proteins. However, these observations do not discount the activation of parallel apoptotic pathways. Similarly, the *in vivo* time course for appearance of cell cycle proteins and cleaved caspase-3 is consistent with our previous *in vivo* work, which demonstrated significant neurodegeneration at 9 h.[2]

Our data are suggestive of and support a role for aberrant cell cycle reentry in ketamine-induced neuroapoptosis. However, several lines of investigation have implicated other neuronal death mechanisms such as excitotoxicity and activation of the extrinsic apoptotic pathway.[21,22] In this study, we show that the knockdown of cyclin D1 by siRNA effectively decreased Bim concentrations and caspase-3 activation, thereby confirming that cyclin D1-regulated cycle cell progression is involved in ketamine-induced neuroapoptosis.

Because anesthetic-induced neurotoxicity is dependent on the relative immaturity of the neurons,[12] we chose to use primary neurons grown for 3 days to assure the immature state of the primary neurons used in the *in vitro* experiments. The distribution of the various NMDA-R subunits changes as neurons mature and may be also involved in age-dependent susceptibility to a variety of neurotoxin.[23] Developmental changes in the NMDA-R subunits mediate distinct physiologic and pharmacologic properties with the embryological NR2 subtype composed primarily of NR2B subunit.[24] This subunit is gradually replaced by the NR2A subunit as the cells assume a mature state after P7.[25] Given these significant differences in NMDA-R composition and pharmacologic

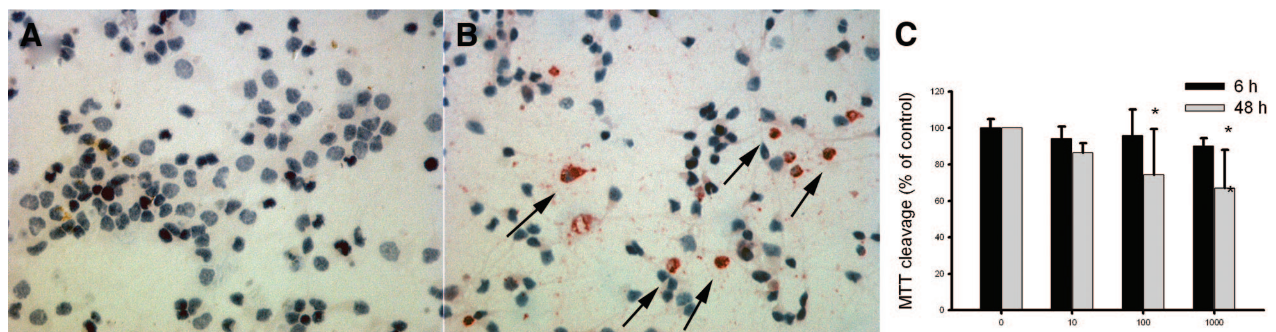

Fig. 9. Ketamine decreases survival of primary neurons in culture. Immunohistochemical staining of primary neurons exposed to saline or ketamine (10 μM) for 48 h shows increased cleaved caspase-3–immunoreactive cells (*arrows*) treated with ketamine (*red*). (*A*) Control and (*B*) 10 μM ketamine. (*C*) Viability primary neurons after incubation with 10 μM ketamine or saline was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *Gray bars* indicate cell viability at 6 h of exposure, and *black bars* indicate 48 h exposure (n = 3, * $P < 0.05$, compared with the control group). Data are presented as mean ± standard error.

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responses, immature neurons are essential in delineating the molecular effects of ketamine-induced developmental neurotoxicity.

In summary, we have shown that ketamine mediates a dose- and duration-dependent activation of cell cycle proteins in developing rodent cortical neurons *in vitro* and *in vivo*. This pathway includes activation of cyclin D1, cdk4, E2F1, and Bim. Bim likely triggers the caspase-3 activation and apoptosis we observe in our model systems. Aberrant cell cycle reentry is not the primary mediator of ketamine-induced neuronal apoptosis. It is just another pathway that is induced by prolonged exposure to ketamine. Knockdown experiments of cyclin D1 resulted in the attenuation of Bim and cleaved caspase-3 levels in neurons treated with ketamine. Our findings are limited to cell cycle activation in neurons. Oligodendrocytes, astrocytes, and microglia enter the cell cycle during periods of proliferation. Astrocytes and microglia are not postmitotic and have the capacity to proliferate, especially in traumatic brain injury.[26] Up-regulation of cell cycle machinery in these glial cells may affect neuronal homeostasis. The effect of ketamine and other anesthetic drugs on these cell types have not been investigated and may provide another avenue for the progression of neurodegeneration after exposure to anesthetic drugs. These data suggest that future investigations into anesthesia-induced neurodegeneration should also focus on signaling pathways that activate cyclin D1 and on its downstream effector proteins.

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