Noxious Stimulation Attenuates Ketamine-induced Neuroapoptosis in the Developing Rat Brain

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ABSTRACT

Background: Ketamine induces neuroapoptosis in neonatal rodents. However, these experimental paradigms were performed without concurrent noxious stimulation, a condition that does not reflect the interaction of anesthesia and surgical stimulation. Noxious stimulation with and without concurrent analgesic drugs has been shown to have divergent patterns of neuronal activation and cell death. We hypothesized that concurrent noxious stimulation would attenuate ketamine-induced caspase-3 activation.

Methods: Postnatal day 7 Sprague-Dawley rat pups were randomized to a 6-h exposure to ketamine with and without peripheral noxious stimulation by intraplantar injection of complete Freund's adjuvant. A cohort of naïve rat pups with and without complete Freund's adjuvant injections served as control subjects. Neuroapoptosis was measured by cleaved caspase-3 expression and terminal deoxynucleotidyl-transferase mediated 2′-deoxyuridine 5′-triphosphate nick end

labeling staining. In order to determine if concurrent noxious simulation altered the expression of cell survival and cell cycle proteins, levels of protein kinase B and glycogen synthase kinase-3β and cyclin D1 were measured.

Results: Ketamine induced a significant increase in cleaved caspase-3 expression and terminal deoxynucleotidyl-transferase mediated 2′-deoxyuridine 5′-triphosphate nick end labeling staining with increases in cyclin D1 levels. Concurrent noxious stimulation with ketamine attenuated caspase-3 activation and maintained cyclin D1 levels. Phosphorylation of protein kinase B and glycogen synthase kinase-3β was not definitively altered under these conditions.

Conclusion: The administration of ketamine with concurrent noxious stimulation results in the attenuation of the neuroapoptotic response. These findings suggest that concurrent surgery and procedural pain attenuates ketamine-induced neuroapoptosis.

P RECLINICAL studies clearly demonstrate that neonatal rats exposed to ketamine, a *N*-methyl-D-aspartic acid antagonist with anesthetic and analgesic properties, leads to widespread neurodegeneration and long-term neurocognitive deficits.[1] Clinical extrapolation of these laboratory reports in rodent models requires experimental conditions equivalent to surgical anesthesia in the human neonate. These experimental paradigms were performed in isolation without concurrent noxious stimulation, a condition that does not reflect the interaction of anesthesia and surgical stimulation.[2] Experimental paradigms of painful and stressful stimuli in newborn rat pups have convincingly demon-

strated abnormalities in long-term behavior and pain perception.[3,4] Fetuses and neonates subjected to pain and stress associated with painful procedures are also at risk for long-term adverse outcomes.[5] Nociceptive stimulation rendered by intraplantar formalin injections or surgical incisions were reported to increase isoflurane-induced neuroapoptosis in neonatal rats.[6] In contrast to this report, concurrent administration of sedative doses of ketamine in a similar neonatal rodent pain model mitigated neuronal cell death and abnormal behavior.[7] Therefore, sedative and anesthetic drugs may have intrinsic neuroprotective and neurotoxic properties in the setting of concurrent noxious stimulation.

Glycogen synthase kinase-3β (GSK-3β) regulates neurogenesis, neuronal polarization, axon growth, and synaptogenesis in the developing central nervous system.[8] GSK-3β activity is suppressed by serine/threonine-specific protein kinase or protein kinase B (AKT) phosphorylation of the GSK-3β serine 9 residue. Increased GSK-3β activity is associated with neurodegenerative and psychiatric conditions. Ketamine-induced neuroapoptosis increased GSK-3β activity, and selective inhibition of GSK-3β attenuates this response in cortical neurons.[9] Experimental models of anesthetic-induced developmental neuroapoptosis are associated with decreased phosphorylated AKT (pAKT), which implicates a regulatory role for the AKT-GSK-3β pathway.[10,11] Furthermore, we previously reported that ketamine-induced aberrant cell cycle reentry by increasing expression of cyclin D1.[12] Glycogen synthase kinase-3β is associated with increased cyclin D1 expression and neuronal cell cycle reentry.[13] The neuroapoptotic effect of high-dose ketamine with concurrent noxious stimulation has not been previously reported. To test the hypothesis that concurrent noxious stimulation will attenuate the ketamine-induced neuroapoptosis in neonatal rat pups, we measured the neuroapoptotic response in the sensory cortex and levels of AKT, GSK-3β, and cyclin D1 in this experimental paradigm.

Materials and Methods

Animals and Reagents

With the approval of the Investigational Review Board and adherence to the Guide for the Care and Use of Laboratory Animals, all experiments utilized Sprague-Dawley postnatal day 7 (P7) rat pups (Charles River Laboratories, Wilmington, MA).[14] Ketamine and complete Freund's adjuvant (CFA) were obtained from commercial sources (Ketalar; Bedford Labs, Bedford, OH, and F5881; Sigma-Aldrich, St. Louis, MO, respectively).

In Vivo Experiments

The treatments were conducted in a temperature-controlled acrylic container maintained at 36.7°C. Similar conditions resulted in core body temperatures between 36.5 and 37.5°C.[15] Each rat pup received five intraperitoneal injections (10 ml/kg each) of either ketamine (2 mg/ml) or vehicle (saline) at 90-m

intervals over 6 h. This dosing regimen is similar to that used in previous investigations utilizing 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.[16] After the administration of the first intraperitoneal dose of saline or ketamine, noxious peripheral stimulation was induced by injecting 0.01 ml of CFA suspended in an oil/saline (9:1) emulsion into the plantar surface of both hindpaws. This is an established experimental model for inducing peripheral noxious stimulation in rodent pain studies and produces tissue inflammation that last several days.[17]

P7 rat pups were randomly assigned into four groups; saline (control); saline with CFA injected in both hind paws (CFA); ketamine followed by CFA injected in both hind paws after the first ketamine dose (KET+CFA); and ketamine (KET). The rat pups were kept from their dam and visually monitored for respiratory effort and activity. After the treatment period, the rat pups were euthanized with intraperitoneal pentobarbital (100 mg/kg). The brains from each group (n = 6 per group) were rapidly isolated and frozen in liquid nitrogen and processed for protein analysis. A second cohort of rat pups (n = 6 per group) were anesthetized with intraperitoneal pentobarbital (100 mg/kg) and immediately perfused with saline followed by 4% paraformaldehyde. The brains were subsequently embedded in paraffin for histologic processing.

Protein Extraction and Western Blotting Analysis

To measure the effect of the various treatment conditions on apoptotic cell death (caspase-3) and AKT, GSK-3β, and cyclin D1, total protein was extracted from flash-frozen brain tissue with radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing protease inhibitor cocktail and 1 mM phenylmethanesulfonylfluoride. Protein concentrations were measured by detergent compatible protein assay (Bio-Rad, Hercules, CA). The equal amounts of protein were boiled in sodium dodecyl sulfate loading buffer (Bio-Rad), resolved on 8–12% polyacrylamide denaturing gels, and transferred to nitrocellulose (Bio-Rad). Antibodies used for Western blotting included rabbit antibodies to cleaved caspase-3 (Cl-Csp3), total and phosphorylated AKT and GSK-3β, cyclin D1, and β-actin (1:2,000, Cell Signaling, Beverly, 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). The densities of the specific protein bands were quantified by Image J 1.42 (National Institutes of Health, Bethesda, MD).

Caspase-3 Immunohistochemistry and TUNEL Staining

The 5 μm cut sections of brain tissues were deparaffinized in xylene and rehydrated through graded alcohol. Endogenous peroxidases were inactivated by immersing the sections in hydrogen peroxide for 10 m, and then were incubated for 10 m with 10% normal goat serum to block nonspecific

binding. The sections were subsequently incubated at 4°C overnight with anticaspase-3 antibody (rabbit polyclonal, immunoglobulin G, 1:100 dilution, (Cell Signaling). Then, the sections were incubated with biotinylated antirabbit immunoglobulin G (Dako, Carpinteria, CA) for 30 m, followed by peroxidase-conjugated streptavidin for 30 m. The chromogenic reaction was developed with 3,3′-diaminobenzidine for 3 m, and all sections were counterstained with hematoxylin. The same protocol was applied to the controls with the omission of the primary antibody.

To determine the cell type that expressed Cl-Csp3, a cohort of brain sections (n = 4 per group) were incubated with rabbit anti-Cl-Csp3 (1:2,500; Cell Signaling). In addition, nuclei were stained with 4–6-diamidino-2-phenylindole (1:10,000, Sigma-Aldrich) and neuron-specific mouse anti-NeuN (1:100; Abcam, Cambridge, MA) antibodies overnight. This was followed by incubating the tissue for 1 h with 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.

Cell apoptosis was also determined using an apoptosis detection kit (Millipore, Serological Corporation, Norcross, GA). This technique is based on the terminal deoxynucleotidyl-transferase mediated 2′-deoxyuridine 5′-triphosphate nick end labeling (TUNEL) assay. Briefly, the brain slices were deparaffinized in xylene and rehydrated through graded alcohol, then washed in phosphate buffered saline, treated with proteinase K (20 μg/ml) (Roche Applied Science, Indianapolis, IN) for 30 m at room temperature, quenched in 3.0% hydrogen peroxide in phosphate buffered saline for 5 m, washed twice, incubated in equilibration buffer for at least 10 s, and labeled with Terminal Deoxynucleotidyl Transferase reaction mix in a humidified box overnight at 4°C. The slices were washed in stop/wash buffer for 10 m and then washed three times in phosphate-buffered saline. The slides were then incubated for 30 m in a solution of anti-digoxigenin conjugate, and colorized with 3, 3′-diaminobenzidine. All sections were counterstained with hematoxylin. Controls consisted of omission of the Terminal Deoxynucleotidyl Transferase reaction mix.

Intraplantar injection of CFA has been shown to activate the anterior cingulate cortex and the somatosensory cortex 1 and 2 regions.[18] In light of this and other reports documenting cellular activation in these specific cortical regions, the number of Cl-Csp3 and TUNEL positive were counted in a double-blinded manner from randomly selected sampling areas from the anterior cingulate cortex and the somatosensory cortex 1 and 2.[7,19]
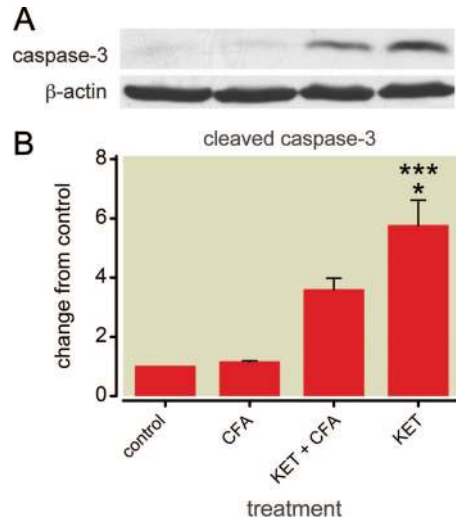

Fig. 1. Concurrent noxious stimulation with CFA attenuates ketamine-induced caspase-3 activation. (*A*) Western blot from postnatal day 7 brain protein lysates demonstrates that intraplantar CFA affects ketamine-induced (*B*) changes in cleaved caspase-3 expression (n = 6, ***$P < 0.01$ compared with control, *$P < 0.05$ ketamine + CFA vs. ketamine). Data are presented as mean ± SE. CFA = complete Freund's adjuvant; KET = ketamine.

Statistical Analysis

Changes in TUNEL and Cl-Csp3 positive cells and protein levels were presented as percentages of control values. Data were expressed as mean ± SE. The differences in these measurements were analyzed by a one-way ANOVA analysis following by *post hoc* Dunnett's test or Newman–Keuls test for individual comparisons. Data analyses were generated and plots were constructed using SPSS for Windows version 18.0 (SPSS Inc., Chicago, IL) and Prism 5 for Mac OS X (GraphPad Software, La Jolla, CA). Statistical significance was set at $P < 0.05$, and all P values were unadjusted for multiple comparisons (two-tailed testing).

Results

Peripheral Noxious Stimulation Attenuates Ketamine-induced Caspase-3 Activation

To investigate if a concurrent noxious stimulus affects caspase-3 activation, we measured Western blot analysis for activated caspase-3, in brain protein lysates from the four treatment groups (fig. 1). Ketamine-treated rat pups had a five- to sixfold increase in Cl-Csp3 and TUNEL positive cells compared with the saline-treated controls (figs. 2 and 3). These data confirm that prolonged exposure to ketamine results in apoptotic cell death. Intraplantar CFA resulted in a minimal increase in Cl-Csp3 and TUNEL staining when compared with the naïve unstimulated pups. Ketamine-treated pups receiving intraplantar CFA had significantly less capase-3 activation when compared with the pups receiving ketamine without peripheral stimulation. A similar distribution pattern was observed in the immunofluorescence microscopy of brain sections stained with
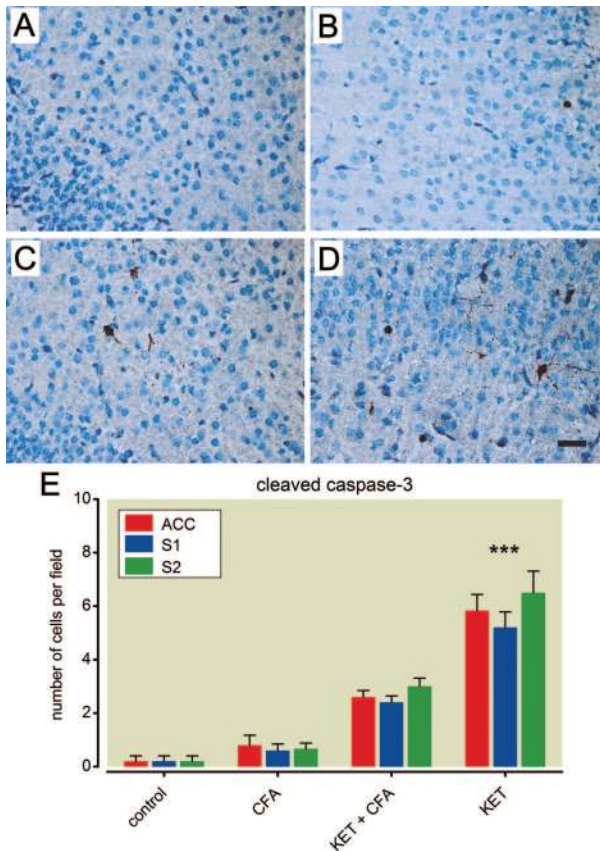
Fig. 2. Concurrent noxious stimulation attenuates ketamine-induced increases in cleaved caspase-3 (Cl-Csp3). Immunohistochemistry with antibody to Cl-Csp3 on S1 cortical sections from postnatal day 7 rat pups in groups (*A*) control, (*B*) CFA, (*C*) ketamine + CFA and (*D*) ketamine (scale bar = 10 μm). (*E*) Quantitation of Cl-Csp3-immunoreactive cells in the ACC, S1, and S2 regions of the postnatal day 7 brain. ****P* < 0.01 compared with saline. Values are means ± SE (n = 6). CFA = complete Freund's adjuvant; KET = ketamine.

Fig. 3. Concurrent noxious stimulation attenuates ketamine-induced increases in TUNEL positive cells. Immunohistochemistry with antibody to cleaved caspase-3 on S1 cortical sections from P7 rat pups (*A*) control, (*B*) CFA, (*C*) KET + CFA and (*D*) KET (scale bar = 10 μm). (*E*) Quantitation of terminal deoxynucleotidyl-transferase mediated 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) positive cells in the ACC, S1, and S2 regions of the P7 brain. ****P* < 0.01 compared with saline. Values are means ± SE (n = 6). CFA = complete Freund's adjuvant; KET = ketamine.

antibodies to Cl-Csp3 (red) and NeuN, a neuron-specific nuclear protein (green) antibodies; most of the Cl-Csp3-positive cells were neurons (fig. 4).

Ketamine Minimally Decreased pAKT and pGSK-3β Levels in the Brain

To determine the effect of ketamine alone and in combination with peripheral noxious stimulation on levels of pAkt and pGSK-3β, we performed Western blot analyses on whole-brain protein lysates from P7 rats from the four treatment groups. This analysis revealed that treatment with ketamine alone minimally decreased levels of the pAKT and pGSK-3β (fig. 5). However, these changes did not definitively account for the significant increase in ketamine-induced Cl-Csp3 levels.

Ketamine Increases Cyclin D1 Expression, Whereas Concurrent Noxious Stimulus Mitigates Response

We previously reported that ketamine mediates a dose- and duration-dependent activation of cell cycle proteins, cyclin
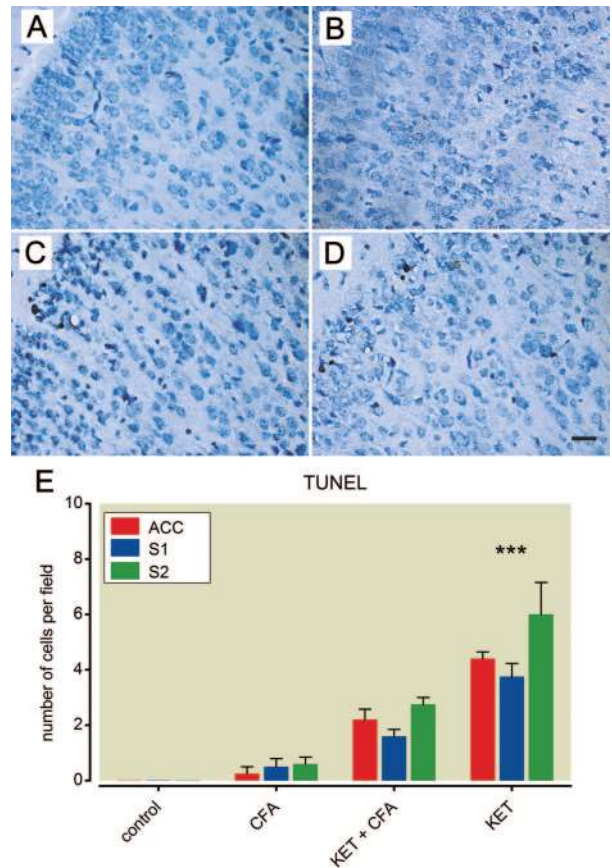
D1, and its downstream effector proteins in developing rat cortical neurons *in vitro* and *in vivo*.[12] Experimental models of neurodegeneration have implicated these cell cycle-related proteins and cell cycle reentry as a potential mechanism for apoptotic cell death. In the present study, we show that ketamine induced an increase in cyclin D1 expression (fig. 6). However, peripheral noxious stimulation with CFA suppressed this response.

Discussion

In this study, we demonstrated that concurrent noxious stimulation with CFA attenuates neuroapoptosis in neonatal rats receiving ketamine. Because the clinical use of ketamine in humans is for the alleviation of pain and distress, these data demonstrate that the phenomenon of anesthetic-induced neuroapoptosis may not be significant in the care of pediatric patients with surgical or procedural pain.
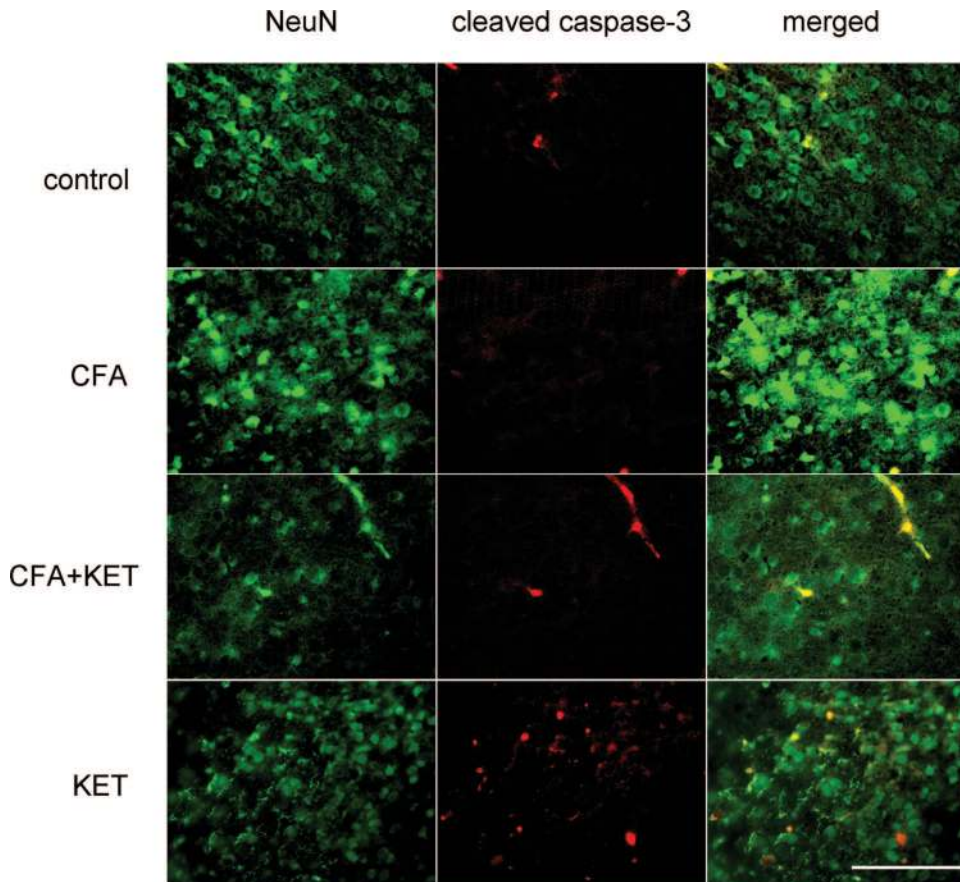
Fig. 4. Caspase-3 activation occurred primarily in neurons. Double staining with antibodies to cleaved caspase-3 (*red*) and antibodies to NeuN, a neuron-specific nuclear protein (*green*), demonstrated that most of the cleaved caspase-3 positive cells were neurons (scale bar = 100 μm). CFA = complete Freund's adjuvant; KET = ketamine.

A major drawback of existing reports on ketamine-induced neurotoxicity is that ketamine was administered to unstimulated animals.[2] This is in contrast to the clinical use of anesthetic agents, which occurs mostly in the presence of painful stimulation. Nociceptive stimuli induced by intraplantar formalin injections or surgical incisions enhanced isoflurane-induced neuroapoptosis in a similar model.[6] This finding is in contrast to previous[7,20] and present observations using ketamine as the anesthetic drug. A comparison of the neuroapoptotic effect of the ketamine and isoflurane in neonatal rhesus macaque revealed that the latter produced fourfold greater increase in neuroapoptosis when compared with former.[21] This discrepancy can be partially explained by the fact that γ-aminobutyric acid agonist effect of isoflurane induces hyperpolarization and excitation of immature neurons, which can lead to excitatory cell death.[22,23] Whereas, prolonged *N*-methyl-D-aspartic acid receptor antagonism by ketamine may lead to depressed neuronal and trophic activity resulting in cell death.[24,25] The cortical activation produced by peripheral noxious stimulation may neutralize the depressant effect of ketamine and result in an attenuated neuroapoptotic response. However, further investigations in other experimental models are required to verify this hypothesis.

The Cl-Csp3 measurements presented in this study show that concurrent noxious stimulation attenuates ketamine-induced neuroapoptosis. Clinical reports clearly demonstrated that repetitive or prolonged pain during the critical periods of brain development in preterm neonates compromises brain structure and function.[26,27] Experimental paradigms of painful stimuli and maternal withdrawal in newborn rat pups have convincingly demonstrated abnormalities in long-term behavior and pain perception.[3,17,28] Furthermore, rat pups subjected to repetitive inflammatory pain with intraplantar injections of formalin developed neuronal excitation and cell death in cortical and subcortical areas of the brain.[7] Concurrent administration of low-dose ketamine (2.5 mg/kg) mitigated this neuronal activation and cell death, thereby demonstrating the neuroprotective effect of ketamine. However, there were no detectable changes in Cl-Csp3 levels.[20] Ketamine-induce neuroapoptosis occurs only with higher dosing regimens (20 mg/kg, every 90 m over 6–9 h).[29,30] Compared with the KET group, the decrease in caspase-3 activation in the KET + CFA group clearly demonstrates that ketamine-induced neuroapoptosis is attenuated by concurrent noxious stimulation.

The effect of peripheral noxious inflammatory stimulation on ketamine-induced neurotoxicity can be attributed by
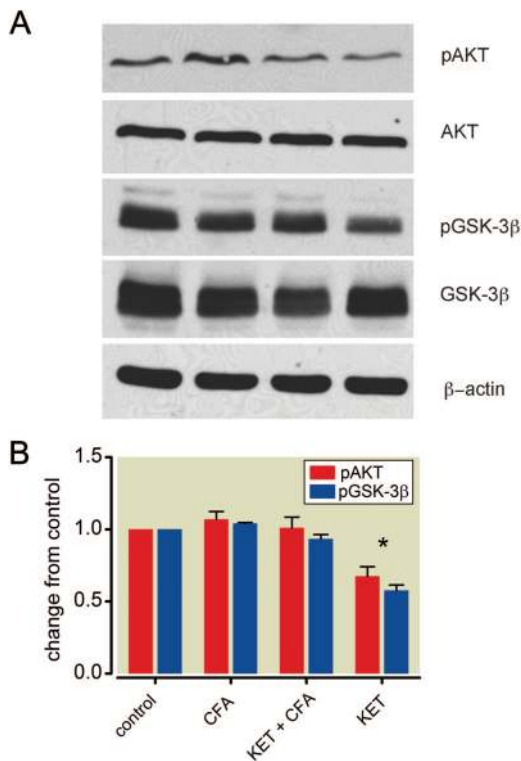
Fig. 5. Levels of phosphorylated protein kinase B and phosphorylated glycogen synthase kinase-3β are minimally affected by ketamine. (*A*) Western blot from postnatal day 7 brain protein lysates demonstrates that ketamine minimally decreased phosphorylation of protein kinase B and (*B*) glycogen synthase kinase-3β (n = 6, *$P < 0.05$ for ketamine *vs.* control and ketamine + CFA). Data are presented as mean ± SE. AKT = protein kinase B; CFA = complete Freund's adjuvant; GSK-3β = glycogen synthase kinase-3β; KET = ketamine; pAKT = phosphorylated protein kinase B; pGSK-3β = phosphorylated glycogen synthase kinase-3β.
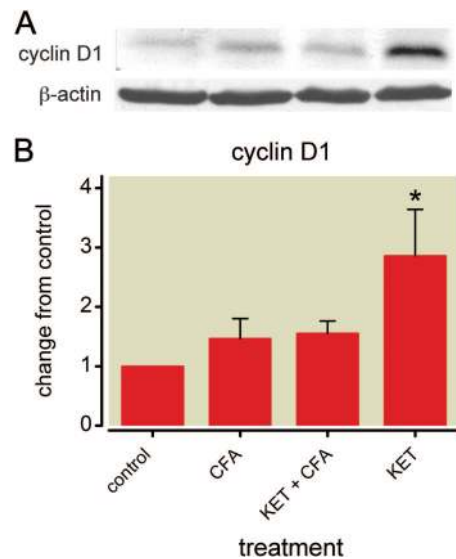

Fig. 6. Concurrent noxious stimulation attenuates ketamine-induced increase in cyclin D1 expression. (*A*) Western blot from postnatal day 7 brain protein lysates demonstrates that intraplantar CFA regulates (*B*) changes in cyclin D1 expression during exposure to ketamine (n = 4, *$P < 0.05$). Data are presented as mean ± SE. CFA = complete Freund's adjuvant; KET = ketamine.

either the afferent input from the inflamed hindpaws or the systemic effects of CFA-induced inflammation. Peripheral inflammation induced by intraplantar injection of CFA induces microglia activation and cytokine production not only in the spinal cord, but also in the brainstem and forebrain in adult rats.[31] Afferent nociceptive input into the sensory cortex leads to activation of neurons and microglia[7,31,32] and opposes ketamine-induced suppression neuronal and trophic activity. Furthermore, intraplantar CFA has been shown to increase hippocampal neurogenesis, whereas repetitive tactile and nociceptive stimulation by cotton swabs and pinpricks respectively did not.[33,34] Likewise, CFA- induced peripheral nociception also produces a systemic T-cell mediated delayed-type hypersensitivity reaction.[35] One experiment that would discriminate the effects of afferent nociceptive input and the systemic effect of intraplantar CFA would be to perform a spinal anesthetic on a cohort of P7 rat pups. However, a spinal anesthetic has a maximal duration of 45 min and the CFA-induced nociception lasts for hours.[36] The current *in vivo* model used in this study is not designed to discriminate between these two processes. Therefore, further

studies would be useful in determining the effect of CFA-induced inflammation in the development of ketamine-induced neuroapoptosis.

Our observations also confirm the effect of increased cyclin D1 expression in ketamine-induced neuroapoptosis.[12] The present observations demonstrate that concurrent CFA-induced noxious peripheral stimulation appears to attenuate this response. Although ketamine has been shown to suppress phosphorylation of AKT in primary cell cultures and unstimulated neonatal mice,[9,10] our findings reveal that ketamine minimally decreased pAKT and pGSK-3β. Furthermore, these changes do not definitively account for the significant alterations in the expression of Cl-Csp3 and cyclin D1. Several lines of investigation have implicated other neuronal cell death mechanisms such as excitotoxicity, modulation of p75 neurotrophin receptor, and intracellular calcium dysregulation,[15,37,38] and a combination of these and other parallel neurodegenerative pathways likely mediate the neurotoxic effect of ketamine on the developing brain.

The spinal cord is also susceptible to the neuroapoptotic effects of anesthetic drugs. Isoflurane has been shown to increase apoptosis spinal cord in neonatal rats.[39] Intrathecal ketamine also produced neuroapoptosis in the spinal cord and gait abnormalities.[40] Although we did not measure caspase-3 activity in this region, it is likely that systemic ketamine will also induce neuroapoptosis in the spinal cord. However, this phenomenon should be examined in future investigations.

Locomotor and neurocognitive deficits have been linked to neonatal exposure to anesthetic drugs.[40–42] Although systemic administration of low-dose ketamine mitigated abnor-

mal pain thresholds and visual-spatial learning in rat pups subjected to repeated inflammatory pain,[7,20] the combined effect of high-dose ketamine and CFA-induced noxious stimulation has not been reported and would be a useful endpoint to examine. Unfortunately, neurocognitive assessments at adulthood of the interaction of ketamine and noxious stimulation on neonatal rats were purposely omitted from this investigation because the CFA-treated pups developed severe inflammation on both hind paws. It was deemed necessary to euthanize the animals at this period in order to minimize persistent pain associated with the CFA injections. Furthermore, the CFA intraplantar injections impaired ambulation, which can affect behavioral assessments that rely on ambulation.

In summary, we have shown that concurrent noxious stimulation attenuates ketamine-induced neuroapoptosis in the neonatal rat brain. Our observations suggest that the administration of drugs that block nociception may not only block surgical stress, but also attenuate the neurodegenerative response in the setting of surgical and procedural anesthesia. These data suggest that ketamine administered with concurrent noxious stimulation (*i.e.*, surgery or procedural pain) may not induce caspase-3 activation as much as the anesthetic in isolation. Future investigations into anesthesia-induced neurodegeneration should examine the role of concurrent pain on signaling pathways and subsequent neurocognitive and locomotor function.

References

1. Loepke AW, Soriano SG: An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. Anesth Analg 2008; 106:1681–707

2. Anand KJ, Soriano SG: Anesthetic agents and the immature brain: Are these toxic or therapeutic? ANESTHESIOLOGY 2004; 101:527–30

3. Johnston CC, Walker CD, Boyer K: Animal models of long-term consequences of early exposure to repetitive pain. Clin Perinatol 2002; 29:395–414

4. Sternberg WF, Scorr L, Smith LD, Ridgway CG, Stout M: Long-term effects of neonatal surgery on adulthood pain behavior. Pain 2005; 113:347–53

5. Anand KJ: Pain, plasticity, and premature birth: A prescription for permanent suffering? Nat Med 2000; 6:971–3

6. Shu Y, Zhou Z, Wan Y, Sanders RD, Li M, Pac-Soo CK, Maze M, Ma D: Nociceptive stimuli enhance anesthetic-induced neuroapoptosis in the rat developing brain. Neurobiol Dis 2012; 45:743–50

7. Anand KJ, Garg S, Rovnaghi CR, Narsinghani U, Bhutta AT, Hall RW: Ketamine reduces the cell death following inflammatory pain in newborn rat brain. Pediatr Res 2007; 62:283–90

8. Hur EM, Zhou FQ: GSK3 signalling in neural development. Nat Rev Neurosci 2010; 11:539–51

9. Takadera T, Ishida A, Ohyashiki T: Ketamine-induced apoptosis in cultured rat cortical neurons. Toxicol Appl Pharmacol 2006; 210:100–7

10. Straiko MM, Young C, Cattano D, Creeley CE, Wang H, Smith DJ, Johnson SA, Li ES, Olney JW: Lithium protects against anesthesia-induced developmental neuroapoptosis. ANESTHESIOLOGY 2009; 110:862–8

11. Sanders RD, Sun P, Patel S, Li M, Maze M, Ma D: Dexmedetomidine provides cortical neuroprotection: Impact on anaesthetic-induced neuroapoptosis in the rat developing brain. Acta Anaesthesiol Scand 2010; 54:710–6

12. Soriano SG, Liu Q, Li J, Liu JR, Han XH, Kanter JL, Bajic D, Ibla JC: Ketamine activates cell cycle signaling and apoptosis in the neonatal rat brain. ANESTHESIOLOGY 2010; 112:1155–63

13. Yeste-Velasco M, Folch J, Trullàs R, Abad MA, Enguita M, Pallàs M, Camins A: Glycogen synthase kinase-3 is involved in the regulation of the cell cycle in cerebellar granule cells. Neuropharmacology 2007; 53:295–307

14. Institute of Laboratory Animal Research Commission on Life Sciences NRC. Guide for the Care and Use of Laboratory Animals. Washington, D.C.: The National Academy Press; 1996

15. Head BP, Patel HH, Niesman IR, Drummond JC, Roth DM, Patel PM: Inhibition of p75 neurotrophin receptor attenuates isoflurane-mediated neuronal apoptosis in the neonatal central nervous system. ANESTHESIOLOGY 2009; 110:813–25

16. Zou X, Patterson TA, Sadovova N, Twaddle NC, Doerge DR, Zhang X, Fu X, Hanig JP, Paule MG, Slikker W, Wang C: Potential neurotoxicity of ketamine in the developing rat brain. Toxicol Sci 2009; 108:149–58

17. Ruda MA, Ling QD, Hohmann AG, Peng YB, Tachibana T: Altered nociceptive neuronal circuits after neonatal peripheral inflammation. Science 2000; 289:628–31

18. Taketa Y, Niikura K, Kobayashi Y, Furuya M, Shimizu T, Narita M, Imai S, Kuzumaki N, Maitani Y, Yamazaki M, Inada E, Iseki M, Suzuki T: Direct evidence for the ongoing brain activation by enhanced dynorphinergic system in the spinal cord under inflammatory noxious stimuli. ANESTHESIOLOGY 2010; 112:418–31

19. Treede RD, Kenshalo DR, Gracely RH, Jones AK: The cortical representation of pain. Pain 1999; 79:105–11

20. Rovnaghi CR, Garg S, Hall RW, Bhutta AT, Anand KJ: Ketamine analgesia for inflammatory pain in neonatal rats: A factorial randomized trial examining long-term effects. Behav Brain Func 2008; 4:35

21. Brambrink AM, Avidan MS, Dissen GA, Creeley C, Olney JW. Neurotoxic impact of isoflurane *versus* ketamine on the neonatal *versus* fetal rhesus macaque brain. Paper presented at: annual meeting of the American Society of Anesthesiologists; October 15–19, 2011; Chicago, IL

22. Ben-Ari Y: Excitatory actions of gaba during development: The nature of the nurture. Nat Rev Neurosci 2002; 3:728–39

23. Edwards DA, Shah HP, Cao W, Gravenstein N, Seubert CN, Martynyuk AE: Bumetanide alleviates epileptogenic and neurotoxic effects of sevoflurane in neonatal rat brain. ANESTHESIOLOGY 2010; 112:567–75

24. Lipton SA, Nakanishi N: Shakespeare in love–with NMDA receptors? Nat Med 1999; 5:270–1

25. Hardingham GE, Bading H: Synaptic *versus* extrasynaptic NMDA receptor signalling: Implications for neurodegenerative disorders. Nat Rev Neurosci 2010; 11:682–96

26. Peterson BS, Vohr B, Staib LH, Cannistraci CJ, Dolberg A, Schneider KC, Katz KH, Westerveld M, Sparrow S, Anderson AW, Duncan CC, Makuch RW, Gore JC, Ment LR: Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. JAMA 2000; 284:1939–47

27. Peters JW, Schouw R, Anand KJ, van Dijk M, Duivenvoorden HJ, Tibboel D: Does neonatal surgery lead to increased pain sensitivity in later childhood? Pain 2005; 114:444–54

28. Fitzgerald M: The development of nociceptive circuits. Nat Rev Neurosci 2005; 6:507–20

29. Hayashi H, Dikkes P, Soriano SG: Repeated administration of ketamine may lead to neuronal degeneration in the developing rat brain. Paediatr Anaesth 2002; 12:770–4

30. Scallet AC, Schmued LC, Slikker W Jr, Grunberg N, Faustino PJ, Davis H, Lester D, Pine PS, Sistare F, Hanig JP: Developmental neurotoxicity of ketamine: Morphometric confirma-

tion, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. Toxicol Sci 2004; 81:364–70

31. Raghavendra V, Tanga FY, DeLeo JA: Complete Freunds adjuvant-induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur J Neurosci 2004; 20:467–73

32. Bie B, Brown DL, Naguib M: Increased synaptic GluR1 subunits in the anterior cingulate cortex of rats with peripheral inflammation. Eur J Pharmacol 2011; 653:26–31

33. Leslie AT, Akers KG, Martinez-Canabal A, Mello LE, Covolan L, Guinsburg R: Neonatal inflammatory pain increases hippocampal neurogenesis in rat pups. Neurosci Lett 2011; 501:78–82

34. Leslie AT, Guinsburg R, Mello LE, Covolan L: Repetitive nociceptive stimuli in newborn rats do not alter the hippocampal neurogenesis. Pediatr Res 2008; 63:154–7

35. Stein C, Millan MJ, Herz A: Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: Alterations in behavior and nociceptive thresholds. Pharmacol Biochem Behav 1988; 31:445–51

36. Yahalom B, Athiraman U, Soriano SG, Zurakowski D, Carpino EA, Corfas G, Berde CB: Spinal anesthesia in infant rats: Development of a model and assessment of neurologic outcomes. ANESTHESIOLOGY 2011; 114:1325–35

37. Slikker W Jr, Paule MG, Wright LK, Patterson TA, Wang C: Systems biology approaches for toxicology. J Appl Toxicol 2007; 27:201–17

38. Wei H: The role of calcium dysregulation in anesthetic-mediated neurotoxicity. Anesth Analg 2011; 113:972–4

39. Sanders RD, Xu J, Shu Y, Fidalgo A, Ma D, Maze M: General anesthetics induce apoptotic neurodegeneration in the neonatal rat spinal cord. Anesth Analg 2008; 106:1708–11

40. Walker SM, Westin BD, Deumens R, Grafe M, Yaksh TL: Effects of intrathecal ketamine in the neonatal rat: Evaluation of apoptosis and long-term functional outcome. ANESTHESIOLOGY 2010; 113:147–59

41. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23:876–82

42. Paule MG, Li M, Allen RR, Liu F, Zou X, Hotchkiss C, Hanig JP, Patterson TA, Slikker W Jr, Wang C: Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. Neurotoxicol Teratol 2011; 33: 220–30